PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room 524
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 27 October 2000 (27.10.00)	
International application No. PCT/EP00/02062	**Applicant's or agent's file reference** PCT/MDC-Anker II
International filing date (day/month/year) 09 March 2000 (09.03.00)	**Priority date** (day/month/year) 09 March 1999 (09.03.99)
Applicant ANKER, Stefan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

26 September 2000 (26.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

BAUMBACH, Fritz
Robert-Rössle-Strasse 10
D-13125 Berlin
ALLEMAGNE

Date of mailing (day/month/year) 21 November 2000 (21.11.00)	
Applicant's or agent's file reference PCT/MDC-Anker II	**IMPORTANT NOTIFICATION**
International application No. PCT/EP00/02062	International filing date (day/month/year) 09 March 2000 (09.03.00)
International publication date (day/month/year) 14 September 2000 (14.09.00)	Priority date (day/month/year) 09 March 1999 (09.03.99)
Applicant MAX-DELBRÜCK-CENTRUM FÜR MOLEKULARE MEDIZIN et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
09 Marc 1999 (09.03.99)	9905315.9	GB	10 Augu 2000 (10.08.00)
09 Marc 1999 (09.03.99)	9905300.1	GB	10 Augu 2000 (10.08.00)
09 Marc 1999 (09.03.99)	9905310.0	GB	10 Augu 2000 (10.08.00)
09 Marc 1999 (09.03.99)	9905307.6	GB	10 Augu 2000 (10.08.00)
09 Marc 1999 (09.03.99)	9905314.2	GB	10 Augu 2000 (10.08.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
Elisabeth KÖNIG

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

10/019452

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
PCT/MDC-Anker		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 02062	09/03/2000	09/03/1999
Applicant		
MAX-DELBRÜCK-CENTRUM FÜR MOLEKULARE MEDIZIN		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   USE OF INHIBITORS OF ENDOTOXIN FOR THE TREATMENT OF CHACHEXIA

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/E[redacted]00/02062

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/575 A61P9/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	SASATOMI K ET AL: "Effects of ursodeoxycholic acid on intrahepatic localization of endotoxin in bile duct-ligated rats and patients with primary biliary cirrhosis." GASTROENTEROLOGY, vol. 110, no. 4 SUPPL., 1996, page A1312 XP000971586 96th Annual Meeting of the American Gastroenterological Association and the Digestive Disease Week;San Francisco, California, USA; May 19-22, 1996 ISSN: 0016-5085 the whole document --- -/--	1-23

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2000	09/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Orviz Diaz, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,Y	SASATOMI KURUMI ET AL: "Abnormal accumulation of endotoxin in biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis." JOURNAL OF HEPATOLOGY, vol. 29, no. 3, 1998, pages 409-416, XP000971565 ISSN: 0168-8278 page 415, right-hand column abstract	1-23
X,Y	SASATOMI K ET AL: "Effects of ursodeoxycholic acid on intrahepatic localization of endotoxin in patients with primary biliary cirrhosis." JOURNAL OF ENDOTOXIN RESEARCH, vol. 3, no. SUPPL. 1, 1996, page 25 XP000971569 Fourth International Endotoxin Society Conference;Nagoya, Japan; October 22-25, 1996 ISSN: 0968-0519 the whole document	1-23
X,Y	POO JORGE L ET AL: "Effects of ursodeoxycholic acid on hemodynamic and renal function abnormalities induced by obstructive jaundice in rats." RENAL FAILURE, vol. 17, no. 1, 1995, pages 13-20, XP000971578 ISSN: 0886-022X the whole document	1-23
X,Y	GREVE J W ET AL: "BILE ACIDS INHIBIT ENDOTOXIN-INDUCED RELEASE OF TUMOR NECROSIS FACTOR BY MONOCYTES AN IN-VITRO STUDY" HEPATOLOGY, vol. 10, no. 4, 1989, pages 454-458, XP000971557 ISSN: 0270-9139 the whole document	1-23
Y	ANKER STEFAN D ET AL: "Elevated soluble CD14 receptors and altered cytokines in chronic heart failure." AMERICAN JOURNAL OF CARDIOLOGY, vol. 79, no. 10, 1997, pages 1426-1430, XP000971579 ISSN: 0002-9149 the whole document	1-23

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	ANKER STEFAN D ET AL: "Soluble CD14 receptors and cytokines in chronic heart failure: Immune activation due to endotoxin?" EUROPEAN HEART JOURNAL, vol. 18, no. ABSTR. SUPPL., 1997, page 297 XP000971584 XIXth Congress of the European Society of Cardiology together with the 32nd Annual General Meeting of the Association of European Paediatric Cardiologists;Stockholm, Sweden; August 24-28, 1997 ISSN: 0195-668X the whole document	1-23
Y	EGERER KARL R ET AL: "Soluble CD14 receptors are increased in cachectic chronic heart failure suggesting a role for endotoxin." CIRCULATION, vol. 94, no. 8 SUPPL., 1996, pages I672-I673, XP000971568 69th Scientific Sessions of the American Heart Association;New Orleans, Louisiana, USA; November 10-13, 1996 ISSN: 0009-7322 the whole document	1-23
Y	SAKISAKA S ET AL: "Biliary secretion of endotoxin and pathogenesis of primary biliary cirrhosis." YALE JOURNAL OF BIOLOGY AND MEDICINE, vol. 70, no. 4, July 1997 (1997-07), pages 403-408, XP000971577 ISSN: 0044-0086 the whole document	1-23

Continuation of Box I.2

The expression "compound that is able to reduce the production, absorption and/or the effect of of an endotoxin" encompasses an extremely large number of possible compounds that might have this mechanism of action, redering a complete search impossible. The search had to be limited to the general mechanism and to the use of bile acids, which are explicitly used in the examples.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

10/019452

REC'D 14 JUN 2001
WIPO PCT

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PCT/MDC-Anker II		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/02062	09/03/2000	09/03/1999
International Patent Classification (IPC) or national classification and IPC A61K31/00		
Applicant MAX-DELBRÜCK-CENTRUM FÜR MOLEKULARE MEDIZIN et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 8 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
26/09/2000	12.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Bochelen, D Telephone No. +49 89 2399 8150

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/02062

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-5,7-9	as originally filed			
6	as received on	31/03/2001	with letter of	26/03/2001

Claims, No.:

1-6	as originally filed			
7-21	as received on	31/03/2001	with letter of	26/03/2001

Drawings, sheets:

1/5-5/5	as received on	31/03/2001	with letter of	26/03/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-21 with regard to industrial applicability; 1-22 (partly), 23: novelty, inventive step.

because:

   ☒ the said international application, or the said claims Nos. 1-21 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
   see separate sheet

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 1-21 (partly).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.
   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability;

citati ns and explanati ns supp rting such statement

1. Statement

Novelty (N)	Yes:	Claims	see separate sheet
	No:	Claims	
Inventive step (IS)	Yes:	Claims	see separate sheet
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	see separate sheet
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The applicant is informed that the opinion regarding novelty, inventive step and industrial applicability will only be formulated with respect of subject-matter which is covered by the search report, i.e. the general mechanism and bile acids (Rule 66(1)(e) PCT).

2. **Claims 1-21** relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3. Reference is made to the following documents:

D1: SASATOMI K ET AL: 'Effects of ursodeoxycholic acid on intrahepatic localization of endotoxin in bile duct-ligated rats and patients with primary biliary cirrhosis.' GASTROENTEROLOGY, vol. 110, no. 4 SUPPL., 1996, page A1312 XP000971586 96th Annual Meeting of the American Gastroenterological Association and the Digestive Disease Week;San Francisco, California, USA; May 19-22, 1996 ISSN: 0016-5085

D2: SASATOMI KURUMI ET AL: 'Abnormal accumulation of endotoxin in biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis.' JOURNAL OF HEPATOLOGY, vol. 29, no. 3, 1998, pages 409-416, XP000971565 ISSN: 0168-8278

D3: SASATOMI K ET AL: 'Effects of ursodeoxycholic acid on intrahepatic localization of endotoxin in patients with primary biliary cirrhosis.' JOURNAL OF ENDOTOXIN RESEARCH, vol. 3, no. SUPPL. 1, 1996, page 25 XP000971569 Fourth International Endotoxin Society Conference;Nagoya, Japan; October 22-

25, 1996 ISSN: 0968-0519

D4: POO JORGE L ET AL: 'Effects of ursodeoxycholic acid on hemodynamic and renal function abnormalities induced by obstructive jaundice in rats.' RENAL FAILURE, vol. 17, no. 1, 1995, pages 13-20, XP000971578 ISSN: 0886-022X

D5: GREVE J W ET AL: 'BILE ACIDS INHIBIT ENDOTOXIN-INDUCED RELEASE OF TUMOR NECROSIS FACTOR BY MONOCYTES AN IN- VITRO STUDY' HEPATOLOGY, vol. 10, no. 4, 1989, pages 454-458, XP000971557 ISSN: 0270-9139

D6: ANKER STEFAN D ET AL: 'Elevated soluble CD14 receptors and altered cytokines in chronic heart failure.' AMERICAN JOURNAL OF CARDIOLOGY, vol. 79, no. 10, 1997, pages 1426-1430, XP000971579 ISSN: 0002-9149

D7: ANKER STEFAN D ET AL: 'Soluble CD14 receptors and cytokines in chronic heart failure: Immune activation due to endotoxin?' EUROPEAN HEART JOURNAL, vol. 18, no. ABSTR. SUPPL., 1997, page 297 XP000971584 XIXth Congress of the European Society of Cardiology together with the 32nd Annual General Meeting of the Association of European Paediatric Cardiologists;Stockholm, Sweden; August 24-28, 1997 ISSN: 0195-668X

D8: EGERER KARL R ET AL: 'Soluble CD14 receptors are increased in cachectic chronic heart failure suggesting a role for endotoxin.' CIRCULATION, vol. 94, no. 8 SUPPL., 1996, pages I672-I673, XP000971568 69th Scientific Sessions of the American Heart Association;New Orleans, Louisiana, USA; November 10-13, 1996 ISSN: 0009-7322

4. The opinion with respect to novelty and inventive is established for the compounds for which a search report has been established, i.e **bile acids** (see point 1 above).

5. **Novelty (Art. 33 (1) and (2) PCT)**:

5.1 The subject-matter of **claims 1-2,** with the limitation set forth in point 5, appears to be new. The prior art does not disclose the use of bile acids, neither for the treatment of cachexia or weight loss (**claim 1**) nor for reducing immune activation in cachexia (**claim 2**). The dependent **claims 3-21** as well fulfill the requirements of Art. 33 (2) PCT.

5.2 Documents D1-D5 disclose the beneficial effect of the bile acid UDCA on the hepatic

endotoxin metabolism in cirrhosis. These documents do not mention the treatment of cachexia or reduction of immune activation in cachexia with bile acids and are thus not relevant for the novelty of the present application.

6. **Inventive step (Art. 33 (1) and (3) PCT)**:

Document D2, which is considered to represent the most relevant state of the art, discloses that the bile acid UDCA has a beneficial effect on the hepatic endotoxin metabolism in cirrhosis (D2: abstract) from which the subject-matter of **claim 1 and 2** differs in that bile acids are used for the treatment of cachexia or to reduce immune activation in cachexia. The prior art discloses that UDCA has beneficial effects on endotoxin metabolism in liver diseases (see D1-D5) and shows a parallel between cachexia and immune activation in chronic heart failure (see D6- D8). There is no indication in the prior art that would prompt a skilled man to consider the use of bile acids for the treatment of cachexia. Therefore, it is considered that the subject-matter of **claims 1 and 2** involves an inventive step. The dependent **claims 3-21** fulfill the requirements of Art. 33 (3) PCT.

7. **Industrial applicability (Art. 33 (1) and (4) PCT)**:

For the assessment of the present **claims 1-21** on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

8. Further to the limitation of the examination to the examples, it should be noted that the substances that fall within the scope of **claims 1-2** are not clearly defined, since no pharmacological criteria is indicated for the selection of potential active compounds, thus rendering the scope of **claims 1-2** obscure. Moreover, the definition

encompasses a large number of compounds and the disclosure is not sufficiently precise for the person skilled in the art to reduce the technical features to practice without undue burden. Therefore, **claims 1-2** do not meet the requirement of Article 6 PCT.

9. **Claims 17-18** do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The compounds that are subject-matter of said claims are defined by the result to be achieved, "..inhibit the response by a cell to endotoxin.." (**claim 17**), "..decrease the cytokine production.." (**claim 18**), which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result are not recited in said claims.

It is important to note that it has never been proposed that ursodeoxycholic acid (UDCA) should be given in patients with weight loss, i.e. cachexia, in patients with liver disease. It has never been proposed that ursodeoxycholic acid (UDCA) could prevent or reverse weight loss, i.e. cachexia, in patients with liver disease. Additionally, it has never been proposed that ursodeoxycholic acid (UDCA) could prevent or reverse weight loss, i.e. cachexia, in patients with chronic obstructive pulmonary disease, chronic renal failure, diabetes, rheumatoid arthritis.

The invention will now be described by reference to the following additional examples and figures.

Example 1:

We have tested the ability of ursodeoxycholic acid (UDCA, FALK Pharma GmbH) and BPi to inhibit LPS-mediated TNF production in whole blood of patients with cachexia.

We studied 4 patients with cachexia due to liver cirrhosis. The patients had all weight loss >7.5% compared to their previous normal weight. In 3 of the 4 patients had a alcoholic aetiology. All patients were studied twice on 2 subsequent days (day "-1" and day "0"), see Figure 1 to 4.

Methods: Heparinized whole blood was diluted 1:10 with medium +/- LPS (50 pg/ml), +/- BPI (1 µg/ml), and +/- UDCA (1 µg/ml – 1 mg/ml) according to the manufactorer's recommendation (Milenia whole blood assay ; DPC Biermann, Bad Nauheim, Germany) and incubated for 4 hours at 37°C. In the supernatant, we assessed concentrations of TNF and IL-6 using the semiautomated Immulite system (DPC-Biermann, Bad Nauheim, Germany).

Results: In patients with cachexia due to liver cirrhosis spontaneous ("Control" data) and LPS-stimulated production of TNF and IL6 is significantly elevated compared to that of healthy subjects, see Figure 5. LPS-stimulated cytokine production was inhibited by UDCA independently of the effects of the ethanol solution. The detailed results are presented in Figure 1 to 4. 1mg/ml UDCA reduced LPS-stimulated TNF production on average by >99% and IL6 production by 97% (ethanol 1% alone on average only by 38% for TNF and 43% for IL6). 100 µg/ml UDCA reduced LPS-stimulated TNF and IL6 production by 42% and 13%, respectively, ethanol 0.1% alone on average only 9% for TNF and IL6 production increased by 18% for ethanol alone).

8. A method according to claim 1 to 4 wherein the compound is bactericidal/permeability increasing protein (BPI).

9. A method according to claim 1 to 4 wherein the compound is, a lipoprotein, for instance, low densitiy lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL), apolipoprotein (a), a lipoprotein mixture.

10. A method according to claim 1 to 4 wherein the treatment is a combination of a compound according claim 7 and claim 9.

11. A method according to claim 1 to 4 wherein the compound is or an antibody capable of binding to endotoxin (lipopolysaccharide; LPS).

12. A method according to claim 1 to 4 wherein the compound is an antibody able to bind to the CD14 receptor.

13. A method according to claim 1 to 4 wherein the compound is a soluble CD14 receptor.

14. A method according to claim 1 to 4 wherein the compound is a drug blocking effectively signaling through toll-like receptors, for instance toll-like receptor 4 and toll-like receptor 2.

15. A method according to claim 1 to 4 wherein the compound is colostrum of human, bovine, or other mamallian origin.

16. A method according to claim 1 to 4 wherein the compound is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS).

17. A method according to claim 1 to 4, and 16 wherein the compound is able to decrease the cytokine production by a cell in response to endotoxin (lipopolysaccharide; LPS).

19. A method according to claim 1, 2 and 16, and 17 wherein the compound is a compound named in claim 5 to 16..A method according to any one of the preceding claims wherein the compound is administered orally.

20. A method according to any one of the preceding claims wherein the compound is administered intravenously.

21. A method according to any one of the preceding claims wherein the compound is administered rectally.

7. A method according to claim 1 to 4 wherein the compound is LPS binding protein.

8. A method according to claim 1 to 4 wherein the compound is bactericidal/permeability increasing protein (BPI).

9. A method according to claim 1 to 4 wherein the compound is, a lipoprotein, for instance, low densitiy lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL), apolipoprotein (a), a lipoprotein mixture.

10. A method according to claim 1 to 4 wherein the treatment is a combination of a compound according claim 7 and claim 9.

11. A method according to claim 1 to 4 wherein the compound is ~~or~~ an antibody capable of binding to endotoxin (lipopolysaccharide; LPS).

~~12. A method according to claim 1 to 4 wherein the compound is or an antibody capable of binding to endotoxin (lipopolysaccharide; LPS).~~

12. ~~13~~. A method according to claim 1 to 4 wherein the compound is an antibody able to bind to the CD14 receptor.

13. ~~14~~. A method according to claim 1 to 4 wherein the compound is a soluble CD14 receptor.

14. ~~15~~. A method according to claim 1 to 4 wherein the compound is a drug blocking effectively signaling through toll-like receptors, for instance toll-like receptor 4 and toll-like receptor 2.

15. ~~16~~. A method according to claim 1 to 4 wherein the compound is colostrum of human, bovine, or other mamallian origin.

16. ~~17~~. A method according to claim 1 to 4 wherein the compound is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS).

17. ~~18~~. A method according to claim 1 to 4, and ~~17~~ 16 wherein the compound is able to decrease the cytokine production by a cell in response to endotoxin (lipopolysaccharide; LPS).

18. ~~19~~. A method according to claim 1, 2 and ~~17~~ 16, and ~~18~~ 17 wherein the compound is a compound named in claim 5 to ~~16~~ 15.

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Figure 1: LPS - Neutralisation by UDCA in whole blood in patient 1

Measurements by Immulite	P1 / -1		P1 / 0		P1 / 1		P1 / 2		P1 / 5	
	TNF	IL6	TNFα	IL6	TNFα	IL6	TNFα	IL6	TNFα	IL6
	pg/ml		pg/ml		pg/ml		pg/ml		pg/ml	
Control, blood alone (Con) Con + 50 *pg/ml* LPS Con + BPI (1µg/ml)	28.7 878 29.7	11.2 573 < 5	70.2 938 15.1	35.2 723 < 5						
Blood with UDCA *1 mg/ml (1% ethanol)* + LPS + UDCA + LPS + Ethanol 1% (no UDCA)	< 4 < 4	12.8 10.7	10. 6.4 648	9.8 6.0 278						
Blood with UDCA *100µg/ml (0.1% ethanol)* + LPS + UDCA + LPS + Ethanol 0.1% (no UDCA)	8.5 813	7.8 153	24.5 692 886	19.7 773 580						
Blood with UDCA *10 µg/ml (0.01% ethanol)* + LPS	38.0 952	11.4 597	93.0 1013	45.7 853						
Plasma levels			9.1	26.7	8.3	28.8	< 4	20.5	5.8	28.1

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Figure 2: LPS - Neutralisation by UDCA in whole blood in patient 2

Measurements by Immulite	P2 / -1		P2 / 0	
	TNFα	IL6	TNFα	IL6
	pg/ml		pg/ml	
Control, blood alone (Con)	29.0	10.5	47.5	25.6
Con + 50 *pg/ml* LPS	731	544	807	587
Con + BPI (1µg/ml)	17.0	<5	9.8	< 5
Blood with UDCA *1 mg/ml (1% ethanol)*	< 4	10.1	< 4	7.8
+ LPS + UDCA	9.7	< 5	< 4	5.4
+ LPS + Ethanol 1% (no UDCA)	569	419	540	405
Blood with UDCA *100µg/ml (0.1% ethanol)*	14.6	7.0	35.2	19.5
+ LPS + UDCA	271	343	459	391
+ LPS + Ethanol 0.1% (no UDCA)	712	546	993	788
Blood with UDCA *10 µg/ml (0.01% ethanol)*	42.4	26.2	54.1	32.0
+ LPS	712	622	744	532
Plasma levels	4.9	6.6	< 4	7.6

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Figure 3: LPS - Neutralisation by UDCA in whole blood in patient 3

Measurements by Immulite	P3 / -1		P3 / 0	
	TNFα	IL6	TNFα	IL6
	pg/ml		pg/ml	
Control, blood alone (Con)	43.1	7.9	52.1	12.9
Con + 50 *pg/ml* LPS	450	378	490	346
Con + BPI (1µg/ml)	16.4	< 5	10.0	< 5
Blood with UDCA *1 mg/ml (1% ethanol)*	6.5	10.4	< 4	9.1
+ LPS + UDCA	< 4	10.3	< 4	10.7
+ LPS + Ethanol 1% (no UDCA)	208	108	288	169
Blood with UDCA *100µg/ml (0.1% ethanol)*	12.1	9.4	21.7	8.4
+ LPS + UDCA	48.0	63.5	241	382
+ LPS + Ethanol 0.1% (no UDCA)	383	285	448	346
Blood with UDCA *10 µg/ml (0.01% ethanol)*	34.7	8.0	39.4	10.7
+ LPS	375	310	468	366
Plasma level	13.0	17.1	10.2	15.9

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Figure ~~12~~ 4: LPS - Neutralisation by UDCA in whole blood in patient 4

Measurements by Immulite	P4 / -1		P4 / 0		P4 / 1		P4 / 2	
	TNFα	IL6	TNFα	IL6	TNFα	IL6	TNFα	IL6
	pg/ml		pg/ml		pg/ml		pg/ml	
Control, blood alone (Con)	34.5	10.4	29.5	10.2	4.7	< 5	4.0	< 5
Con + 50 *pg/ml* LPS	224	98.4	172.0	77.5	156	88.9	100	46.8
Con + BPI (1µg/ml)	18.1	< 5	33.5	7.1	< 4	< 5	< 4	< 5
Blood with UDCA *1 mg/ml (1% ethanol)*	< 4	8.2	< 4	8.2	< 4	< 5	< 4	8.8
+ LPS + UDCA	< 4	6.6	< 4	9.5	< 4	5.6	< 4	6.3
+ LPS + Ethanol 1% (no UDCA)	132	48.0	98.5	68.4	66.9	35.6	49.5	26.5
Blood with UDCA *100µg/ml (0.1% ethanol)*	83.7	49.6	44.5	60.0	4.2	< 5	< 4	< 5
+ LPS + UDCA	242	257	55.8	61.0	89.7	36.6	39.9	34.6
+ LPS + Ethanol 0.1% (no UDCA)	154	130	136.0	159	141	82.7	120	81.0
Blood with UDCA *10 µg/ml (0.01% ethanol)*	42.3	13.6	174	72.0	10.9	< 5	5.2	< 5
+ LPS	188	80.2	278	166	163	88.7	95.6	53.4
Plasma levels	8.1	< 5	< 4	< 5	< 4	< 5	4.7	< 5

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Figure 8: LPS - Neutralisation by UDCA in whole blood of 4 healthy subjects

Measurements by Immulite	Control D		Control J		Control Ch		Control F	
	TNF	IL6	TNFα	IL6	TNFα	IL6	TNFα	IL6
	pg/ml		pg/ml		pg/ml		pg/ml	
Control, blood alone (Con)	6.7	<5	4.6	<5	14	8	15.4	<5
Con + 50 *pg/ml* LPS	294	301	456	380	486	300	589	487
Con + BPI (1µg/ml)	<4	<5	6.9	<5	6.9	<5	8	<5
Blood with UDCA *1 mg/ml (1% ethanol)*	4.8	<5	6	6.5	<4	8.5	<4	<5
+ LPS + UDCA	<4	5.6	<4	<5	6.4	9.1	<4	<5
+ LPS + Ethanol 1% (no UDCA)	126	119	315	286	407	318	430	408
Blood with UDCA *100µg/ml (0.1% ethanol)*	10.6	<5	14	<5	42.7	46.3	16	<5
+ LPS + UDCA	114	114	41.3	20.4	49.6	306	397	419
+ LPS + Ethanol 0.1% (no UDCA)	265	230	221	263	599	375	569	414
Blood with UDCA *10 µg/ml (0.01% ethanol)*	8.5	<5	8.3	<5	13.7	9	12.3	<5
+ LPS	279	248	432	358	617	400	600	499

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(71) Applicant *(for all designated States except US)*: **MAX-DELBRÜCK-CENTRUM FÜR MOLEKULARE MEDIZIN** [DE/DE]; Robert-Rössle-Strasse 10, D-13125 Berlin (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ANKER, Stefan** [DE/DE]; Hedrichplatz 25, D-10367 Berlin (DE). **COATS, Andrew** [AU/GB]; 105 A Cadogan Gardens, London SW3 2RF (GB). **VOLK, Hans-Dieter** [DE/DE]; Rathausstrasse 11, D-10178 Berlin (DE). **SCHUMANN, Ralf, Reiner** [DE/DE]; Buchenallee 104, D-16341 Zepernick (DE). **PLAUTH, Mathias** [DE/DE]; Paderbornerstrasse 9, D-10709 Berlin (DE). **LOCHS, Herbert** [DE/DE]; Helmstedter Strasse 8, D-10717 Berlin (DE).

(74) Agent: **BAUMBACH, Fritz**; Robert-Rössle-Strasse 10, D-13125 Berlin (DE).

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(54) Title: USE OF INHIBITORS OF ENDOTOXIN FOR THE TREATMENT OF CHACHEXIA

(57) Abstract: The present invention relates to therapy and the use of agents in the therapy of cachexia and wasting syndromes due to diseases other than congestive heart failure. Cachexia occurs in a number of other chronic diseases, like liver cirrhosis, chronic obstructive pulmonary disease, chronic renal failure, diabetes, rheumatoid arthritis. Cachexia and weight loss are linked to inflammatory processes and they are linked to increased mortality and/or morbidity. Cytokine activation is a potential causal mechanism for the development of cachexia also in these other diseases. The invention describes a method of treating or ameliorating body wasting or cachexia in a patient with liver cirrhosis, chronic obstructive pulmonary disease, chronic renal failure, diabetes, rheumatoid arthritis in a patient. The method comprises administering to the patient an effective amount of a compound that is able to reduce the production, absorption and/or the effect of an endotoxin (lipopolysaccharide; LPS). The invention describes also a method of treating, preventing or ameliorating endotoxin-mediated immune activation in body wasting or cachexia in a patient with liver cirrhosis, chronic obstructive pulmonary disease, chronic renal failure, diabetes, rheumatoid arthritis. The method comprises administering to the patient an effective amount of a compound that is able to reduce the production, absorption and/or the effect of an endotoxin (lipopolysaccharide; LPS).

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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (1[2])

(54) Method for Assaying for a Substance that Affects an Sh2-Phosphorylated Ligand Regulatory System

(72) Pawson, Anthony - Canada ;

(73) Mount Sinai Hospital Corporation - Canada ;

(57) 12 Claims

filed as U.S. Pat 5,352,660

Canada

ABSTRACT OF THE DISCLOSURE

A method for assaying a medium for the presence of a substance that affects an SH2-phosphorylated ligand regulatory system. The method employs an SH2-like domain or a subdomain thereof and a phosphorylated ligand. The phosphophorylated ligand is capable of interacting with the SH2-like domain or a subdomain thereof to form an SH2-phosphorylated ligand complex. The SH2-like domain or subdomain and/or the phosphorylated ligand are present in a known concentration. The SH2-like domain or a subdomain thereof and the phosphorylated ligand are incubated with a substance which is suspected of affecting an SH2-phosphorylated ligand regulatory system. The method is carried out under conditions which permit the formation of the SH2-phosphorylated ligand complex. SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand are assayed. The invention also relates to an isolated SH2-phosphorylated ligand complex; a method of using an isolated SH2-like domain or a subdomain thereof to screen for phosphorylated ligands which are active in an SH2-phosphorylated ligand regulatory system; a method of using an isolated SH2-like domain or a subdomain thereof to regulate the interaction of a signalling protein with a related phosphorylated ligand; and a pharmaceutical composition comprising an isolated SH2-like domain or a subdomain thereof for use as an agonist or antagonist of the interaction of a signalling protein with a related phosphorylated ligand.

RB&P File No. 3153-048

Title: Method for Assaying for a Substance that Affects an SH2-Phosphorylated Ligand Regulatory System

FIELD OF THE INVENTION

The invention relates to a method for assaying a medium for the presence of a substance that affects an SH2-phosphorylated ligand regulatory system; an isolated SH2-phosphorylated ligand complex; a method of using an isolated SH2-like domain or a subdomain thereof to screen for phosphorylated ligands; a method of using an isolated SH2-like domain or a subdomain thereof to regulate the interaction of a signalling protein with a related phosphorylated ligand; and a pharmaceutical composition comprising an isolated SH2-like domain or a subdomain thereof.

BACKGROUND OF THE INVENTION

A common mechanism by which growth factors regulate cellular proliferation and differentiation is through transmembrane receptors with inducible protein-tyrosine kinase activity (Ullrich and Schlessinger, Cell 61, 203 (1990); Pawson and Bernstein, Trends Gen. 6, 350 (1990)). Indeed the mitogenic effects of growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) absolutely require the tyrosine kinase activity of their receptors (Chen et al., Nature 328, 820 (1987); Honneger, Mol. Cell. Biol. 7, 4568 (1987); Williams, Science 243, 1564 (1989)). Growth factors induce receptors to cluster, which is followed by intermolecular tyrosine phosphorylation of the oligomerized receptors (Yarden and Schlessinger, Biochemistry 26, 1434 (1987); Böni-Schnetzler and Pilch, Proc. Natl. Acad. Sci. U.S.A. 84, 7832 (1987); Heldin et al., J. Biol. Chem. 264, 8905 (1989)). Autophorphorylation of the PDGF receptor (PDGFR) is important both for its

subsequent interactions with substrates and for the induction of DNA synthesis (Kazlauskas and Cooper, Cell 58, 1121 (1989); Coughlin et al., Science 243, 1191 (1989); Kazlauskas et al., Science 247, 1578 (1990)).

A second group of tyrosine kinases, for which Src, Fps, and Abl are the prototypes, are entirely intracellular (Pawson, Oncogene 3, 491 (1988)). In the case of the Src-like tyrosine kinase Lck, which is specifically expressed in T cells, the NH_2-terminal region of the kinase associates with the short cytoplasmic tails of the cell adhesion molecules CD4 and CD8 (Veillette et al., Cell 55, 301 (1988); Rudd et al., Proc. Natl. Acad. Sci. U.S.A. 85, 5190 (1988); Shaw et al., Cell 59, 627 (1989)). In addition, Src and the related kinases Fyn and Yes physically associate with, and are phosphorylated by, the β-PDGFR (Kypta et al., Cell 62, 481 (1990)). PDGF stimulation is associated with a three- to five-fold increase in Src kinase activity, which may serve to amplify the tyrosine kinase signal (Kypta et al., Cell 62, 481 (1990); Ralston and Bishop, Proc. Natl. Acad. Sci. U.S.A. 82, 7845 (1985); Gould and Hunter, Mol. Cell. Biol. 8, 3345 (1988)). Hence, the Src-like kinases also appear to participate in signal transduction.

Many structural alterations have been documented for both receptor-like and cytoplasmic tyrosine kinases, which induce constitutive tyrosine kinase activity and simultaneously activate oncogenic potential (Ullrich and Schlessinger, Cell 61, 203 (1990); Pawson and Bornstein, Trends Gen. 6, 350 (1990); Hunter and Cooper, Annu. Rev. Biochem. 54, 897 (1985)). The biological activities of transforming tyrosine kinases, like their normal counterparts, are generally dependent on their kinase activity.

After stimulation with PDGF or EGF several proteins become physically associated with, and phosphorylated by, the activated PDGFR or EGF receptor (EGFR). A number of these receptor-binding proteins have

been identified, including phosphoinositide-specific phospholipase C(PLC)-γ1 (Margolis et al, Cell 57 1101 (1989); Meisenhelder et al., ibid., p. 1109), $p21^{ras}$ GTPase-activating protein (GAP) (Kazlauskas et al., Science 247, 1578 (1990); Kaplan et al., ibid. 61, 121 (1990)), phosphatidylinositol (PI) 3'-kinase (PI3K) (Kazlauskas and Cooper, Cell 58, 1121 (1989); Coughlin et al., Science 243, 1191 (1989)), Src and Src-like tyrosine kinases (Kypta et al., Cell 62, 481 (1990)), and Raf (Morrison et al., ibid. 58, 649 (1989); Morrison et al., Proc. Natl. Acad. Sci. U.S.A. 85, 8855 (1988)). These associated proteins are likely targets of receptor activity.

PLC-γ1 is one of several PLC isoforms that cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to the second messengers diacylglycerol and inositol triphosphate, which in turn stimulate protein kinase C and raise intracellular calcium (Rhee et al., Science 244, 546 (1989)). PDGF stimulates PI turnover in cells where PLC-γ1 is the principal PLC isoform (Margolis et al., Cell 57, 1101 (1989); Meisenhelder et al., ibid., p. 1109), and overexpression of PLC-γ1 enhances the accumulation of inositol phosphates in response to PDGF (Margolis et al., ibid. 248, 607 (1990)). Thus, PLC-γ may couple PDGF stimulation to the breakdown of PIP_2.

PI3K phosphorylates the inositol ring of PI in the D-3 position (Whitman et al, Nature 332, 644 (1988)). PI3K activity is associated with a variety of activated tyrosine kinases and correlates with the presence of a tyrosine phosphorylated 85-kilodalton (kD) protein (p85) (Kaplan et al., Cell 50, 1021 (1987); Courtneidge and Heber, ibid., p. 1031; Fukui and Hanafusa, Mol. Cell. Biol. 9, 1651 (1989)). Purified PI3K is a heterodimeric complex that contains p85 and a 110-Kd protein (p110) (Carpenter et al., J. Biol. Chem. 265, 19704 (1990)). The purified p85 subunit has no detectable PI3K activity, but binds tightly to activated PDGFR or EGFR in vitro. PDGF stimulation induces accumulation of PI-3,4-P_2 and

PI-3,4,5-P_3, confirming that PI3K is regulated by tyrosine kinases in vivo (Auger et al., ibid. 57, 167 (1989)).

GAP stimulates the ability of $p21^{ras}$ (Ras) to hydrolyze GTP to GDP (guanosine diphosphate) (B. Margolis et al., ibid, 248, 607 (1990)) and thereby acts as a negative regulator by returning Ras from the active GTP-bound state to the inactive DGP-bound conformation. GAP interacts with the presumed effector region of $p21^{ras}$ (Adari et al., (1988) Science 240, 518-521; Cales, (1988) Nature (London) 332, 548-551) suggesting that it might also be the Ras target or might modify the association of $p21^{ras}$ with its target.

Raf is a protein-serine/threonine kinase that complexes with the PDGFR after PDGF stimulation, although it is unclear whether this is a direct interaction (Morrison et al., ibid. 58, 649 (1989); Morrison et al., Proc. Natl. Acad. Sci. U.S.A. 85, 8855 (1988)). In addition to these proteins, several unidentified polypeptides bind to activated PDGFR (Kazlauskas and Cooper, Cell 58, 1121 (1989); Coughlin et al., Science 243, 1191 (1989); Kazlauskas and Cooper, EMBO J. 9, 3279 (1990)).

The proteins that associate with activated growth factor receptors have quite distinct enzymatic properties and are structurally unrelated within their catalytic domains. However, with the exception of Raf they share conserved noncatalytic domains termed Src homology (SH) regions 2 and 3 (see Figure 1 where 3 represents SH-3 domain; Ras GA the Ras GTPase activating region of GAP; PLC the catalytic sequences of PLC-γ1; gag, retroviral coat protein sequence; CYS, cysteine rich domain of Vav; LEU, leucine-rich region of Vav). The SH2 domain is a sequence of ~100 amino acids, originally identified in the vFps and vSrc cytoplasmic tyrosine kinases by virtue of its effects on both catalytic activity and substrate phosphorylation (T. Pawson,

Oncogene 3, 491 (1988) and I. Sadowski et al., Mol. Cell. Biol. 6, 4396 (1986)).

An SH2 sequence has also been identified in the v-Crk oncoprotein, which complexes with several tyrosine phosphorylated proteins in crk-transformed cells (Mayer et al., Nature 332, 272 (1988); Mayer and Hanafusa, Proc. Natl. Acad. Sci. U.S.A. 87, 2638 (1990)). Most SH2-containing proteins also contain a motif, SH3, which is found independently in several cytoskeletal proteins and may mediate interactions with the cytoskeleton (Pawson, Oncogene 3, 491 (1988); Mayer et al., Nature 332, 272 (1988); Mayer and Hanafusa, Proc. Natl. Acad. Sci. U.S.A. 87, 2638 (1990); Rodaway et al., Nature 342, 624 (1989); Drubin et al., Nature 343, 288 (1990)).

SUMMARY OF THE INVENTION

The present inventors have determined by direct evidence that SH2 domains can mediate the interactions of diverse signalling proteins including cytoplasmic protein tyrosine kinases, $p21^{ras}$ GTPase-activating protein (GAP), phospholipase $C\gamma$ and the V-Crk oncoprotein, with a related set of phosphotyrosine ligands, including the epidermal growth factor (EGF) receptor. In particular, the present inventors found that in Src-transformed cells GAP forms heteromeric complexes, notably with a highly tyrosine phosphorylated 62-kDa protein (p62). The stable association between GAP and p62 can be specifically reconstituted in vitro by using a bacterial polypeptide containing only the N-terminal GAP SH2 domain. The efficient phosphorylation of p62 by the v-Src or v-Fps tyrosine kinases depends, in turn, on their SH2 domains and correlates with their transforming activity. In lysates of EGF-stimulated cells, the N-terminal GAP SH2 domain binds to both the EGF receptor and p62. Fusion proteins containing GAP or v-Crk SH2 domains complex with similar phosphotyrosine proteins from src-transformed or EGF-stimulated cells but with different efficiencies. SH2 sequences, therefore, form autonomous domains that direct

signalling proteins, such as GAP, to bind specific phosphotyrosine-containing polypeptides. By promoting the formation of these complexes, SH2 domains are ideally suited to regulate the activation of intracellular signalling pathways by growth factors.

The inventors have most importantly found that the SH2 domains of cytoplasmic signalling proteins such as PLCγ1, GAP, Src and Crk are sufficient for in vitro binding to activated growth factor receptors. In particular, the inventors found that the SH2 domains of PLCγ1 synthesized individually in bacteria formed high affinity complexes with the epidermal growth factor (EGF)- or platelet derived growth factor (PDGF)-receptors in cell lysates, and bound synergistically to activated receptors when expressed together as one bacterial protein. In vitro complex formation was dependent on prior growth factor stimulation and was competed by intracellular PLCγ1. Similar results were obtained for binding of GAP SH2 domains to the PDGF-receptor. The isolated SH2 domains of other signalling proteins, such as $p60^{src}$ and Crk, also bound activated PDGF-receptors in vitro.

The use of a specialized non-catalytic domain to direct formation between protein kinases and their presumptive targets is unprecedented.

The finding that SH2 domains mediate the interactions of phosphorylated ligands with signalling proteins which regulate pathways that control gene expression, cell division, cytoskeletal architecture and cell metabolism permits the identification of substances which affect the interactions of phosphorylated ligands with signalling proteins and accordingly may be used in the treatment of conditions involving perturbation of signalling pathways. For example, it may be possible to identify substances which block an SH2-containing oncoprotein, or SH2 signalling protein or the actions of deregulated tyrosine kinases which interact with specific SH2 signalling proteins, and that may be useful in

preventing transformation activity. In particular, in the case of cancers where there are deregulated tyrosine kinases, such as thyroid, breast carcinoma, stomach cancer and neuroblastoma, the method of the invention would permit the identification of substances which interfere with the binding of SH2 signalling proteins and the deregulated tyrosine kinase. In the case of cancers such as chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL), an SH2-containing onceprotein interacts with a signalling protein which is autophosphorylated on serine resulting in transformation. The method of the present invention could be used to identify substances which interfere with the interaction and which may be useful in the treatment of CML and ALL.

Therefore, the present invention relates to a method for assaying a medium for the presence of a substance that affects an SH2-phosphorylated ligand regulatory system comprising providing an SH2-like domain or a subdomain thereof, and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof to form an SH2-phosphorylated ligand complex, said SH2-like domain or subdomain thereof and/or said phosphorylated ligand being present in a known concentration, and incubating with a substance which is suspected of effecting an SH2-phosphorylated ligand regulatory system, under conditions which permit the formation of said SH2-phosphorylated ligand complex, and assaying for said SH2-phosphorylated ligand complex, free SH2-like domain or subdomain thereof, or non-complexed phosphorylated ligand.

In a preferred embodiment of the invention, a method is provided for assaying a medium for the presence of an agonist or antagonist substance of an SH2-phosphorylated ligand regulatory system comprising providing an SH2-like domain or a subdomain thereof, and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof to form

an SH2-phosphorylated ligand complex, said SH2-like domain or subdomain thereof and/or said phosphorylated ligand being present in a known concentration, and incubating with a suspected agonist or antagonist substance, under conditions which permit the formation of said SH2-phosphorylated ligand complex, and assaying for said SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand.

The invention also provides a method for screening for antagonists that inhibit the effects of agonists of an SH2-phosphorylated ligand regulatory system. Thus, a substance that competes for the same binding site on the phosphorylated ligand or on the SH2-like domain or a subdomain thereof may be assayed.

The invention further provides an isolated SH2-phosphorylated ligand complex comprising an SH2-like domain or a subdomain thereof and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof.

The invention still further provides a method of using an isolated SH2-like domain or a subdomain thereof to screen for phosphorylated ligands which are active in an SH2-phosphorylated ligand regulatory system.

The invention also relates to a method of using an isolated SH2-like domain or a subdomain thereof to regulate the interaction of a signalling protein with a related phosphorylated ligand and a pharmaceutical composition comprising an isolated SH2-like domain or a subdomain thereof for use as an agonist or antagonist of the interaction of a signalling protein with a related phosphorylated ligand.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1 shows the locations of SH2 domains of signalling proteins;

Figure 2 shows the amino acid sequences of several known SH2 domains;

Figure 3 shows the locations of SH2 and SH3 domains in signalling and transforming proteins and in TrpE fusion proteins;

Figure 4 shows the immunoblots and autoradiograms of TrpE fusion proteins that were mixed with lysates of normal Rat-2 cells or v-src transformed Rat-2 cells;

Figure 5 shows the immunoblots of immobilized TrpE fusion proteins that were mixed with lysates of serum-starved Rat-1 cells overexpressing human EGFR that were stimulated with 0 or 80 nM EGF (A) and immunoblots with anti-EGFR antibodies of nitrocellulose filters containing duplicate samples of those in A (B);

Figure 6 shows immunoblots with anti-phosphotyrosine antibodies of total cell lysates, or anti-GAP immunoprecipitates from Rat-2 cells expressing either wild type $P130^{gag\text{-}fps}$ (v-fps), or mutant $P130^{gag\text{-}fps}$ with a glu^{832}->lys amino acid substitution in the SH2 domain (K-832) (A) and immunoblots with anti-phosphotyrosine antibodies of anti-GAP immunoprecipitates, or total cell lysates from Rat-2 cells expressing wt v-src, or the SRX5, SHX13 or XD6 v-src mutants, or containing empty vector;

Figure 7 shows the locations of SH2 and SH3 domains in TrpE fusion proteins;

Figure 8 shows immunoblots of immobilized TrpE fusion proteins that were mixed with lysates of Rat-1 cells overexpressing EGFR (A) and lysates from serum-starved Rat-2 cells stimulated with 75nM BB-PDGF(B);

Figure 9 shows immunoblots of immobilized TrpE fusion proteins that were mixed with serum-starved Rat-2 cells stimulated with 75nM BB-PDGF; and

Figure 10 shows immunoblots of immobilized TrpE fusion proteins mixed with Rat-2 cells that overexpress PLCγ1.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned the invention relates to a method for assaying a medium for the presence of a substance that effects an SH2-phosphorylated ligand regulatory system comprising providing an SH2-like domain or a subdomain thereof, and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof to form an SH2-phosphorylated ligand complex, said SH2-like domain or subdomain and/or said phosphorylated ligand being present in a known concentration, and incubating with a substance which is suspected of effecting an SH2-phosphorylated ligand regulatory system, under conditions which permit the formation of said SH2-phosphorylated ligand complex, and assaying for said SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand.

In a preferred embodiment a method is provided for assaying a medium for the presence of an agonist or antagonist substance of an SH2-phosphorylated ligand regulatory system comprising providing an SH2-like domain or a subdomain thereof, and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof to form an SH2-phosphorylated ligand complex, said SH2-like domain or subdomain and/or said phosphorylated ligand being present in a known concentration, and incubating with a suspected agonist or antagonist substance, under conditions which permit the formation of said SH2-phosphorylated ligand complex, and assaying for said SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand.

The invention further provides an isolated SH2-phosphorylated ligand complex comprising an SH2-like domain or a subdomain thereof and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof.

The invention still further provides a method of using an isolated SH2-like domain or a subdomain thereof to screen for phosphorylated ligands which are active in an SH2-phosphorylated ligand regulatory system.

The invention also relates to a method of using an isolated SH2-like domain or a subdomain thereof to regulate the interaction of a signalling protein with a related phosphorylated ligand and a pharmaceutical composition comprising an isolated SH2-like domain or a subdomain thereof for use as an agonist or antagonist of the interaction of a signalling protein with a related phosphorylated ligand.

The term "SH2-like domain or a subdomain thereof" refers to a sequence which is substantially homologous to a Src homology region 2 (SH2 region), or a subdomain of an SH region preferably a conserved region of an SH region. The Src homology region is a noncatalytic domain of ~100 amino acids which was originally identified in the Vfps and Vsrc cytoplasmic tyrosine kinases by virtue of its effects on both catalytic activity and substrate phosphorylation (T. Pawson, Oncogene 3, 491 (1988) and I. Sadowski et al., Mol. Cell. Biol. 6, 4396 (1986)).An SH2 sequence has also been identified in the v-Crk oncoprotein, which complexes with several tyrosine phosphorylated proteins in crk-transformed cells (Mayer et al., Nature 332, 272 (1988); Mayer and Hanafusa, Proc. Natl. Acad. Sci. U.S.A. 87, 2638 (1990)).

The sequences of several known SH2 domains are aligned in Figure 2. In Figure 2, residues that are conserved within at least three subfamilies of SH2 domains are capitalized and shaded. Residues that are conserved within one or two groups are capitalized. Residues that are poorly or not at all conserved are in lowercase. Invariant residues are indicated by asterisks. Conserved basic amino acids that might participate in interactions with phosphotyrosine are arrowed. Conserved motifs I to

V are indicated by solid lines, whereas the connecting variable regions i to iv are indicated by broken lines. The suffix N indicates the more NH_2-terminal SH2 domain of PLC-γ, GAP or p85 whereas C indicates the more COOH-terminal domain. The SH2 domain of two isoforms of PLC-γ (γ1 and γ2) and p85 (α and β) are shown (Otsu et al., Cell 65, 91 (1991)). Sequences were aligned by eye. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

An inspection of the aligned SH2 sequences reveals the presence of five well-conserved sequence motifs (designated I to V in Figure 2), which are separated by more variable sequence elements (i to iv). The variable regions generally contain one or more glycine or proline residues, suggesting that they form turns or hinges that connect the conserved subdomains.

The identification of SH2-like domains may be accomplished by screening a cDNA expression library with a phosphorylated ligand with high affinity to SH2 domains (e.g. the autophosphorylated COOH-terminal tail to the EGFR) to isolate cDNAs for SH2 proteins. One could use PCR (Wilks, A.F., Proc. Natl. Acad. Sci. U.S.A. Vol. 86, pp. 1603-1607, March 1989) or low stringency screening (Hanks, S.K., Proc. Natl. Acad. Sci. U.S.A. Vol. 84, pp 388-392, January 1987) with SH2 specific probe.

The term "phosphorylated ligand" refers to a polypeptide or peptide that is capable of interacting with an SH2-like domain or a subdomain thereof, and includes phosphotyrosine, and phosphoserine/phosphothreonine-containing peptides or polypeptides. Examples of ligands which may be utilized in the method of the invention are the SH2 binding sites on transmembrane receptors with inducible protein-tyrosine kinase activity and cytoplasmic tyrosine phosphorylated proteins.

It will be appreciated that the selection of an SH2-like domain or subdomain thereof and a phosphorylated ligand in the method of the invention will depend on the nature and expected utility of the substance to be assayed.

The phosphorylated ligand is preferably synthetically constructed having regard to the interaction of the phosphorylated ligand with a particular SH2 domain.

The term "SH2-phosphorylated ligand regulatory system" used herein refers to the interactions of an SH2-like domain or a subdomain thereof and a phosphorylated ligand and includes the binding of an SH2-like domain or a subdomain thereof to a phosphorylated ligand or any modifications to the SH2-like domain or a subdomain thereof or to the phosphorylated ligand associated therewith, to form an SH2/ligand complex thereby activating a series of regulatory pathways that control gene expression, cell division, cytoskeletal architecture and cell metabolism. Examples of such regulatory pathways are the GAP/Ras pathway, the pathway that regulates the breakdown of polyphosphoinositides through phospholipase C (PLC), and the Src/tyrosine kinase pathway.

The term "signalling protein" used herein includes cytoplasmic protein tyrosine kinases, $p21^{ras}$ GTPase-activating protein (GAP), phospholipase Cγ and the V-Crk oncoprotein, phosphatidylinositol (PI) 3'-kinase (PI3K), Src and Src-like tyrosine kinases, and Raf.

The invention may be used to assay for a substance that affects the interaction of an SH2-like domain or a subdomain thereof and a phosphorylated ligand, preferably a suspected agonist or antagonist. The agonist or antagonist may be an endogenous physiological substance or it may be a natural or synthetic drug.

The SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand in the method of the invention may be isolated by conventional isolation techniques, for

example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof.

The assaying for SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand in the method of the invention may be carried out using known methods. To facilitate the assay of the components, antibody against the SH2-like domain or a subdomain thereof or the phosphorylated ligand, or a labelled SH2-like domain or a subdomain thereof, or a labelled phosphorylated ligand may be utilized.

The SH2 domain or subdomain thereof or phosphorylated ligand may be used to prepare monoclonal or polyclonal antibodies. Conventional methods can be used to prepare the antibodies. As to the details relating to the preparation of monoclonal antibodies reference can be made to Goding, J.W., Monoclonal Antibodies: Principles and Practice, 2nd Ed., Academic Press, London, 1986.

An SH2 domain or subdomain thereof or phosphorylated ligand may be labelled with various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive phosphorous ^{32}P, iodine I^{125}, I^{131} or tritium.

Radioactive labelled materials may be prepared by radiolabeling with ^{125}I by the chloramine-T method (Greenwood et al, Biochem. J. 89:114, 1963), the lactoperoxidase method (Marchalonis et al, Biochem. J. 124:921, 1971), the Bolton-Hunter method (Bolton and

Hunter, Biochem. J. 133:529, 1973 and Bolton Review 18, Amersham International Limited, Buckinghamshire, England, 1977), the iodogen method (Fraker and Speck, Biochem. Biophys. Res. Commun. 80:849, 1978), the Iodo-beads method (Markwell Anal. Biochem. 125:427, 1982) or with tritium by reductive methylation (Tack et al., J. Biol. Chem. 255:8842, 1980).

Known coupling methods (for example Wilson and Nakane, in "Immunofluorescence and Related Staining Techniques", W. Knapp et al, eds, p. 215, Elsevier/North-Holland, Amsterdam & New York, 1978; P. Tijssen and E. Kurstak, Anal. Biochem. 136:451, 1984) may be used to prepare enzyme labelled materials. Fluorescent labelled materials may be prepared by reacting the material with umbelliferone, fluorescein, fluorescein isothiocyanate, dichlorotriazinylamine fluorescein, dansyl chloride, derivatives of rhodamine such as tetramethyl rhodamine isothiocyanate, or phycoerythrin.

The SH2 domain or subdomain thereof or phosphorylated ligand used in the method of the invention may be insolubilized. For example, the SH2 domain or subdomain thereof or phosphorylated ligand may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc

The insolubilized SH2 domain or subdomain thereof or phosphorylated ligand may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The invention also provides a method for screening for antagonists that inhibit the effects of

agonists of an SH2-phosphorylated ligand regulatory system. Thus, a substance that competes for the same binding site on the phosphorylated ligand or on the SH2-like domain or a subdomain thereof is screened for.

It will be understood that the substances that can be assayed using the methods of the invention may act on one or more of the SH2-binding site on the phosphorylated ligand or the ligand-binding site on the SH2-like domain or subdomain thereof, including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

The following materials and methods were utilized in the investigations outlined in Examples 1 and 2:

Antibodies

Polyclonal rabbit antibodies against human GAP residues 171-448 or phosphotyrosine were raised and affinity-purified, as described in Ellis, C. et al (1990) Nature (London) 343, 377-381 and Kamps, M.P. & Sefton, B.M. (1988) Oncogene 2, 305-315. Anti-trpE rabbit antiserum was raised against a 37 Kda protein containing the N-terminal 323 residues encoded by the Escherichia coli trpE protein. Affinity-purified rabbit anti-phosphotyrosine antibodies were prepared as described in Kamps, M.P. & Sefton, B.M. (1988) Oncogene 2, 305-315. Antibodies directed against a peptide corresponding to residues 1176-1186 of the human EGF-R (Honegger, A.M. et al., (1989) Proc.Natl.Acad.Sci. USA 86, 925-929) were utilized.

Cell Culture

Growth conditions, ^{32}Pi labeling, EGF treatment, and immunoprecipitation of R1hER (obtained from M. Weber,

University of Virginia, Charlottesville), Rat-2, and Rat-2 cells expressing v-src or v-fps genes were as described in Declue, J. & Martin, G.S. (1989) J. Virol. 63, 542-554; Koch, V.A. et al. (1989) Mol. Cell. Biol. 9, 4131-4140; and Ellis, C. et al (1990) Nature (London) 343,377-381.

Complex Formation with Bacterial trpE Fusion Proteins

Restriction fragments from human GAP, bovine PLC_γ, or v-crk CDNAS were subcloned into PATH bacterial TrpE expression vectors, using both natural and engineered restriction sites (Ellis, C. et al (1990) Nature (London) 343, 377-381). Fifty ml cultures of E. coli RR1 with the parental PATH expression plasmid, or a derivative encoding one of the various TrpE fusion proteins were grown and induced with indole acrylic acid as described in Moran, F. et al (1988) Oncogene 3, 665-672. Cells were washed with 1 ml of 50 mM Tris-HCl, pH 7.5, 10% (wt./vol.) sucrose followed by a 2 minute centrifugation at 15,000 x g. The cells were resuspended in 1 ml of ice-cold PLCLB (50 Mm HEPES, Ph 7.0/150 Mm NaCl/10% glycerol/1% Triton X-100/1.5 Mm $MgCl_2$/1 Mm EGTA/100 Mm NaF/10 Mm $NaPP_i$/1 Mm Na_3VO_4/1 Mm phenyl/methylsulfonyl fluoride/aprotinin and leupeptin each at 10 µg/ml) sonicated 6 times for 10 seconds each and clarified by centrifugation at 15,000 x g for 15 minutes. Sonication and all subsequent steps were done at 4°C. Supernatants were incubated with 40 µl of anti-trpE serum and 30 µl of protein A-Sepharose beads. After being gently mixed for 90 minutes, the immune complexes were washed three times with HNTG buffer (20 Mm HEPES, Ph 7.0 150 Mm NaCl, 0.1% Triton X-100, 10% glycerol, 1 Mm Na_3VO_4) and divided into four equal aliquots. Similar amounts of the different TrpE fusion protein were detected in these immune complexes by immunoblotting with anti-TrpE antiserum.

For in vitro binding experiments, approximately 5×10^6 non-radioactive or ^{32}P-labelled cells were lysed in 1 or 2 ml PLCLB and clarified as described below. One ml of clarified lysate was incubated with one aliquot of an

anti-trpE immune complex. After mixing by gentle inversion for 90 minutes at 4°C, the immune complexes were recovered by centrifugation, washed three times with HNTG, resuspended in 40 µl of SDS sample buffer and heated at 100°C for 3 minutes.

Immunoblotting

Cell lysates (prepared as in Koch, C.A. et al (1989) 9, 4131-4140; 25 µg of protein per lane), immunoprecipitates, and bacterial complexes were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose in a semi-dry blotting apparatus at 0.8 $Ma.cm^{-2}$ for 60 minutes. Blots were analyzed by autoradiography (^{32}P-labelled samples) or were blocked and then probed with anti-EGFR antiserum (1:200 dilution) or antiphosphotyrosine antibodies as described in Koch, C.A. et al (1989) Mol. Cell. Biol. 9, 4131-4140. Antiphosphotyrosine blots of whole-cell lysates were probed with 10 µ Ci of ^{125}I-labelled protein A (2-10 µ Ci/µg; 1 Ci = 37 GBq; New England Nuclear), whereas all other blots were probed with 5 µ Ci of high-specific-activity ^{125}I-labelled protein A (35 µ Ci/µg, Amersham). Blots were exposed to Kodak XAR film at -75°C with an intensifying screen.

Example 1

GAP and Crk SH2 Domains Bind a Related Set of Phosphotyrosine-containing Proteins.

The disposition of SH2 and SH3 domains within several signalling and transforming proteins is shown in Figure 1. GAP was initially used to test whether these regions might be involved in protein-protein interactions. Different regions of GAP were expressed in bacteria as TrpE-GAP fusion proteins joined to a 37 -Kda TrpE protein (Figure 3). The fusion proteins contained the following residues: TrpE-GAP-SH2, human GAP 171-448; TrpE-GAP-SH2(N), GAP 178-278; TrpE-GAP-SH2(C), GAP 348-445; TrpE-GAP-C, GAP 670-1047; TrpE-V-Crk, $P47^{gag\text{-}crk}$ 206-327; TrpE-

PLC_γ, bovine $PLC_\gamma 1$ 956-1291.3=SH3 domain; GA=GTPase activating region of GAP.

TrpE-GAP-SH2 contains almost precisely the two GAP SH2 domains and the intervening SH3 sequence. In contrast, TrpE-GAP-C contains the C-terminal half of GAP, including all residues required to stimulate $p21^{ras}$ GTPase activity (Marshall, M.S. et al (1989) EMBO. J. 8, 1105-1110). As controls, the TrpE protein by itself and a TrpE-PLC_γ fusion protein containing C-terminal PLC_γ catalytic sequences were used. These TrpE fusion proteins were immunoprecipitated with anti-TrpE antiserum.

To investigate whether these polypeptides could form specific complexes with proteins from src-transformed cells, the immunoprecipitates were incubated with a lysate of Rat-2 v-src cells (Figure 4A Lanes 5-8) and with lysates of normal Rat-2 fibroblasts (Figure 4A Lanes 1-4) and analyzed for associated proteins by immunoblotting with anti-phosphotyrosine antibodies. Phosphotyrosine bound to TrpE-GAP-SH2 from Rat-2 v-src cells (Lane 9) were also compared directly with an anti-GAP immunoprecipitate from the same lysate (Lane 10).

TrpE, TrpE-PLC_γ and TrpE-GAP-C which lack SH2 sequences, did not retain any phosphotyrosine-containing proteins from the Rat-2 v-src lysate. However, TrpE-GAP-SH2 bound a 62 KDA tyrosine phosphorylated protein, as well as variable amounts of a 130 Kda protein (Figure 4A). The 62 Kda protein co-migrated with p62 immunoprecipitated with anti-GAP antibodies from Rat-2 v-src cells.

As a more direct test of their binding activities, the TrpE fusion proteins were incubated with lysate of Rat-2 v-src cells that had been metabolically labelled with $^{32}P_i$ (Lanes 11-13). A lysate from $^{32}P_i$-labeled Rat-2 v-src cells was also incubated with anti-GAP antibodies (Lane 14). Precipitated ^{32}P labelled proteins were visualized by autoradiography (right panel). Exposure time was 3 hours, except for lane 14 (18 hours). Again, TrpE-GAP-SH2 specifically bound a 62 Kda

phosphoprotein that comigrated with GAP-associated p62 (Figure 4A). The same result was obtained using ^{32}P-labelled v-fps-transformed cells. Tryptic phosphopeptide analysis confirmed the identity of the 62-Kda SH2-binding protein as p62. p62 is not obviously related to $p60^{src}$, and lacks detectable in vitro protein kinase activity. The 130 Kda protein that bound the TrpE-GAP-SH2 may correspond to a protein (p130) whose phosphorylation by activated $p60^{src}$ requires the Src SH2 domain, with which it complexes in vivo (Reynolds, A.B. et al. (1989) Mol. Cell. Biol. 9, 3951-3958 and Lau, A. F. (1986) Virology 151, 86-99).

Immobilized TrpE (Figure 4B), TrpE-GAP-SH2(N) (Figure 4B), TrpE-GAP-SH2(C) (Figure 4B), TrpE-GAP-SH2 (Figure 4B) and TrpE-v-Crk (Figure 4B) were incubated with lysates from Rat-2 v-src cells (Figure 4B) or normal Rat-2 Cells (Figure 4B). For comparison, anti-GAP immunoprecipitations (Figure 4B) were made from the same cell lysates. Samples were analyzed by immunoblotting with anti-phosphotyrosine antibodies and ^{125}I-Protein-A. Autoradiography was for 16 hours (lanes 1-6) or 3 days (lanes 7-14).

The binding sites for p62 and p 130 were more precisely ascribed to the N-terminal SH2 domain of GAP (GAP-SH2(N), Figure 3) which efficiently bound p62 and p130 from Rat-2 v-src cells (Figure 4B).

To investigate whether these tyrosine phosphorylated proteins might be more general ligands for SH2-containing proteins similar experiments were done with a TrpE-v-Crk fusion protein (Figure 3). TrpE-v-Crk also bound two phosphotyrosine-containing proteins when incubated with a Rat-2 v-src lysate, which likely correspond to p62 and p130 (Figure 4B). TrpE-v-Crk bound p130 more efficiently than did TrpE-GAP-SH2, and also associated with a distinct 70 kDa tyrosine phosphorylated protein (p70). In lysates of normal Rat-2 cells TrpE-GAP-SH2 bound a small amount of p62, whereas TrpE-v-Crk formed more readily detectable complexes with p130 and p70

(Figure 4B). It is of interest that phosphotyrosine-containing proteins of this size are associated with P47$^{gag-crk}$ in v-crk-transformed chicken embryo fibroblasts, and bind bacterial v-Crk in lysates of v-crk-transformed cells (Mayer, B.J. et al (1988) Nature London) 332, 272-275; Mayer, B.J. et al (1988) (Cold Spring Harbor Symp. Quant. Biol. 53, 907-914; Mayer, B.J. & Hanafusa, H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2638-2642). These results indicate that the GAP and Crk SH2 domains have distinct but overlapping binding specificities. They bind common phosphotyrosine-containing ligands, but apparently with different efficiencies.

Example 2

The N terminal GAP SH2 Domain Binds Activated EGF Receptor In Vitro.

GAP has been implicated in the response to growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGR), and shown to form a physical complex with the PDGF-receptor. Therefore the binding activity of TrpE-GAP bacterial proteins in lysates of Rat-1 cells expressing the human EGF-receptor (EGF-R)(~2.5 x 10^5 per cell) was investigated.

Serum-starved (for 48 hours) Rat-1 cells overexpressing human EGF-receptors were stimulated with 0 (Figure 5 lanes 9 to 16), or with 80 nM EGF (lanes 1 to 8) for 5 minutes at 37oC. Cells lysates were mixed with the indicated TrpE bacterial fusion proteins, immunobilized with anti-TrpE antibodies (lanes 1-5,9-13), or immunoprecipitated with anti-GAP (lanes 6,14), anti-EGF-R (lanes 7,15) or anti-phosphotryosine (lanes 8,16) antibodies. Complexes and immunoprecipitates were washed and analyzed by western blotting with antiphosphotryosine antibodies. Nitrocellulose filters containing duplicate samples of those in were immunoblotted with anti-EGF-R antibodies (Figure 5B).

No phosphotyrosine-containing proteins associated with immobilized TrpE fusion proteins before

EGF stimulation (Figure 5A), or with TrpE-GAP-C following addition of EGF. However, TrpE-GAP-SH2, TrpE-GAP-SH2(N) and TrpE-v-Crk precipitated two tyrosine phosphorylated proteins from lysates of EGF-stimulated cells, with mobilities of 62 and 180 kDa (Figure 5A). The 62 kDa protein comigrated with p62 precipitated from the EGF-stimulated lysate with anti-GAP antibodies. The 180 kDa band comigrated with the EGF-R immunoprecipitated from the same lysate, was recognized by anti-EGF-R antibodies on an immunoblot (Figure 5B), and was phosphorylated on tyrosine in an in vitro kinase reaction. These data show that the 180-kDa protein is the EGF-R and that its association with SH2 domains is clearly dependent on prior EGF stimulation (Figure 5B). TrpE-v-Crk bound the EGF-R more effectively than the GAP SH2 fusion proteins, but was less efficient in p62-binding (Figures 5A and B, lane 5)

Example 3

Fps and Src SH2 Domains Are Required for Tyrosine Phosphorylation of p62 and GAP

p62 is rapidly and abundantly phosphorylated by activated v-Src and v-Fps tyrosine kinases (Ellis, C., et al. (1990) Nature (London) 343, 377-381). The v-Fps SH2 domain, and Glu-832 in particular have been previously implicated in recognition of a 62-kDa protein whose phosphorylation correlates with transformation (Koch, C.A. et al. (1989) Mol. Cell. Biol. 9, 4131-4140). Therefore, an investigation was carried out to determine whether this substrate corresponds to p62, which displays an affinity for SH2 domains in vitro (see Example 1). In particular, total cell lysates, or anti-GAP immunoprecipitates from Rat-2 cells expressing either wild type $P130^{gag\text{-}fps}$ (v-fps), or a glu^{832}->lys amino acid mutant (K-832) were analyzed by immunoblotting with anti-phosphotryosine antibodies.

Direct comparison revealed that GAP-associated p62, precipitated with anti-GAP antibodies from cells transformed by wild type (wt) v-fps, comigrated with the prominent SH2-dependent 62-kDa substrate identified in the

whole cell lysate. Furthermore, little phosphotyrosine-containing p62 could be detected in anti-GAP immunoprecipitates from cells expressing a v-Fps mutant with a substitution of lysine for Glu-832 in the SH2 domain (Figure 6A). GAP itself is a relatively poor substrate for $P130^{gag\text{-}fps}$ (Ellis, C. et al. (1990) Nature (London) 343, 377-381); prolonged exposure revealed that GAP tyrosine phosphorylation also depends on the v-Fps SH2 domain.

A series of in-phase linker-insertion and deletion mutations constructed in v-src has yielded several mutants that have relatively high levels of $p60^{v\text{-}src}$ kinase activity, but are poorly transforming in Rat-2 cells (DeClue, J. & Martin, G.S. (1989) J. Virol. 63, 542-554). The XD6 and SHX13 mutants have alterations within highly conserved regions of the v-Src SH2 domain. XD6 has a deletion of residues 149-174, and the SHX 13 mutation inserts Arg-Ala after residue 228. In contrast, the SRX5 mutation replaces the codon for the tyr^{416} autophosphorylation site in the catalytic domain with codons for Ser-Arg-Asp.

Anti-GAP immunoprecipitates (Figure 6B, left panel), or total cell lysates (Figure 6B, middle panel) from Rat-2 cells expressing wild type v-src, or the SRX5, SHX13 or XD6 v-src mutants, or containing empty vector, were analyzed by immunoblotting with anti-phosphotyrosine antibodies. The focus forming activities of the v-src mutants on Rat-2 cells relative to wt are indicated (DeClue, J. & Martin, G.S. (1989) J. Virol. 63, 542-554). In addition, Rat-2 v-src cells were metabolically labelled with ^{32}Pi for 2 hours, followed by immunoprecipitation with anti-phosphotyrosine or anti-GAP antibodies. These immunoprecipitates were separated by gel electrophoresis, transferred to immunoblots and subjected to autoradiography (Figure 6B, right panel).

Rat-2 cells expressing these v-src mutants contained similar levels of GAP and $p60^{v\text{-}src}$ compared with

wild type v-src-transformed cells. However, anti-GAP immunoprecipitations showed that the tyrosine phosphorylation of GAP-associated p62, and of GAP itself, was greatly decreased in cells expressing the SHX13 and XD6 v-src SH2 mutants, correlating with their particularly low Rat-2 transforming activity (Figure 6B). In contrast, the SRX5 autophosphorylation site mutant has an intact SH2 domain, retains 13% of wild type transforming activity on Rat-2 cells, and still gives appreciable phosphorylation of p62 and GAP. Unlike p62, which is minor but highly phosphorylated protein, p190 contains relatively little phosphotyrosine but it is a major GAP-binding protein (Ellis, C. et al (1990) Nature (London) 343,377-381). p190 tyrosine phosphorylation was not affected by the v-src or v-Fps SH2 mutations and hence, does not require the tyrosine kinase SH2 domain and does not correlate with transformation. Binding of tyrosine phosphorylated p190 to GAP SH2 domains or C-terminal region in vitro was not observed, possibly because all the available p190 is already associated with GAP in cell lysates.

Example 4

SH2 domains of PLCγ1 synthesized in bacteria bind synergistically in vitro to activated EGF- and PDGF-receptors.

The following materials and methods were utilized in the example:

Restriction sites were introduced on either side of SH2 coding sequences in the cDNA's for bovine PLCγ1 and human GAP with oligonucleotide-directed mutagenesis (Kunkel, et al., Methods Enzymol. 154, 367 (1987)). For each individual SH2 domain an Sph I site was created at the 5' end and an Nhe I site at the 3' end. These Sph I-Nhe I fragments were cloned into a pATH bacterial trpE expression vector whose multiple cloning site had been modified to contain unique Sph I and Nhe I sites. For fusions that contained both SH2 domains, the Sph I site of

the NH_2- terminal SH2 domain and the Nhe I site of the COOH-terminal SH2 domain were used for the excision, Src and Crk fusion proteins utilized natural restriction sites. The resulting fusion proteins contained the NH_2-terminal 323 amino acids of TrpE and retained the desired reading frame for PLCγ1 or GAP.

Cultures of E. coli RR1 with pATH expression plasmids were grown, induced, and lysed as described above in Example 1.The TrpE fusion proteins were recovered from the supernatants by immunoprecipitation with polyclonal anti-TrpE antiserum immobilized on protein A-Sepharose beads. Immune complexes were washed, aliquoted, flash-frozen, and stored at -70°C until mixed with mammalian cell lysates. Starved or growth factor-stimulated rat fibroblasts (~5 x 10^6) were lysed in 2 ml of lysis buffer (50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). Clarified mammalian cell lysate (1 ml) was mixed with immobilized bacterial fusion protein by gentle inversion for 90 min at 4°C. Complexes were recovered by centrifugation, washed three times with HNTG buffer (20 mM Hepes pH 7.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4), and analyzed by immunoblotting with anti-P.Tyr or anti-receptor as described in Kazlauskas et al. Science 247, 1578 (1990); Koch et al. Mol. Cell. Biol. 9, 4131 (1989); and Ellis et al., Nature 343, 377 (1990). To ensure that the different TrpE fusion proteins were present in similar amounts in the immune complexes incubated with the mammalian cell lysates, duplicate samples for anti-P.Tyr and anti-EGF-R immunoblotting were probed with an anti-TrpE monoclonal antibody. Equivalent amounts of the various TrpE fusion proteins were detected.

To investigate the possibility that enzymes such as PLCγ and GAP associate directly with activated tyrosine kinase receptors by virtue of their SH2 domains,

restriction sites were introduced into the complementary DNA (cDNA) for bovine PLCγ1, which allowed the precise excision of the NH_2- terminal and COOH-terminal SH2 domains (SH2[N] and SH2[C]), either alone or together (See detailed method described above and Fig.7). The individual SH2 domains, or the two SH2 domains together (SH2[N+C]) were introduced into a bacterial expression vector (pATH) and expressed as TrpE fusion proteins in Escherichia coli. These proteins were isolated from bacterial lysates by immunoprecipitation with antibodies to TrpE (anti-TrpE) attached to Sepharose beads (See detailed method described above).

The immobilized bacterial proteins (parental TrpE or the indicated TrpE-PLCγ1 bacterial fusion proteins) were incubated with lysates of Rat-1 cells that overexpressed the human EGF-R (R1hER), which had been serum-starved for 48 hours (Figure 8, lanes 11 to 15) or stimulated for 5 min at 37°C with 80 nM EGF (Figure 8, lanes 1 to 10). Complexes were washed, resolved on 8.25% SDS-polyacrylamide gels, and analyzed by immunoblotting with either anti(α)-P.Tyr (Figure 8, lanes 1 to 5) or anti-EGF-R (Figure 8, lanes 6 to 15) followed by I^{125}-labelled protein A. Autoradiography was for 18 hours. Immobilized TrpE or TrpE-PLCγ1 fusion proteins were also incubated with lysates from Rat-2 cells that were serum-starved for 48 hours (Figure 8, lanes 11 to 15) or stimulated for 5 min at 37°C with 75 nM BB-PDGF (Figure 8, lanes 1 to 10). Samples were resolved on 6% SDS-polyacrylamide gels and analyzed by immunoblotting with either anti-P.Tyr (Figure 8, lanes 1 to 5) or anti-PDGF-R (Figure 8, lanes 6 to 15).

The TrpE-PLC-SH2[N] fusion protein complexed specifically with a 180-kilodalton (kD) P.Tyr-containing protein in lysates of EGF-stimulated cells. Immunoblotting of duplicate samples with antibodies to the EGF-R confirmed that this protein was the EGF-R and showed that its in vitro association with the PLCγ1 SH2[N] domain

was EGF-dependent (Figure 8). The PLCγ1 SH2[N] domain was more efficient than the SH2[C] domain in its ability to bind the EGF-R. Interestingly, the fusion protein that contained both NH_2- and COOH-terminal SH2 domains bound two to four-fold more EGF-R in EGF-stimulated cell lysates than could be accounted for by the two individual SH2 domains. The PLCγ1 SH2 domains therefore functioned synergistically in binding to the activated EGF-R. Very similar results were obtained for interactions of the PLCγ1 SH2 domains with the PDGF-R (Fig. 8). The PLCγ1 SH2[N] domain bound the PDGF-R in lysates of cells treated with the BB homodimeric form of PDGF but not in lysates of unstimulated cells. As observed for the EGF-R, the PLCγ1 SH2[C] domain alone was inefficient in binding activated PDGF-R, but bound synergistically with the SH2[N] domain when both domains were expressed as one bacterial protein (Fig. 8).

Within the SH2 domain, there are motifs that are particularly highly conserved. For example the NH_2-terminal tryptophan is invariant, and most SH2 domains start with the consensus W(Y,F)(H,F)GK (Koch et al. Mol. Cell. Biol. 9, 4131 (1989)). (Note Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.) These residues may have been conserved because they are important in the interactions of SH2-containing proteins with activated growth factor receptors. A TrpE fusion protein that contained both PLCγ1 SH2 domains, with the exception that the first four residues of SH2[N] (W-F-H-G) were deleted (PLC$\triangle$SH2-SH2-3) was expressed and its association with phosphotyrosine containing proteins in cell lysates using the techniques described above was investigated. The fusion protein showed a modest ability to bind activated EGF- or PDGF-R (Fig. 8, lanes 5 and 10) that was equivalent to the SH2[C]

domain alone, indicating that the removal of the four residues weakened binding activity.

Example 5

Binding of TryE fusion proteins that contain the GAP, Src, or Crk SH2 domains to PDGF-R in lysates of PDGF-stimulated Rat-2 cells.

The following procedure was used to investigate binding of TryE fusion proteins that contain GAP, Src, or Crk SH2 domains to PDGF-R in lysates of stimulated Rat-2 cells. Serum- starved Rat-2 cells were stimulated for 5 min at 37°C with 75 nM BB-PDGF, lysed, and mixed with the indicated immobilized TrpE bacterial fusion proteins. Complexes were washed, resolved on 7.5% SDS-polyacrylamide gels and analyzed by immunoblotting with anti-P.Tyr (8 hour exposure) or with anti-PDGF-R (18 hour exposure).

Because GAP also associates with the PDGF-R, experiments were carried out using bacterial GAP SH2 sequences (see Figure 7). The GAP SH2[N] domain bound the PDGF-R in a lysate of PDGF-stimulated cells (Fig. 9), but not in unstimulated cells. The GAP SH2[C] domain exhibited much weaker PDGF-R-binding activity. However, the two SH2 domains together (GAP-SH2[N + 3 + C] bound the receptor threefold more efficiently than expected from their individual binding activities (Fig. 9, lanes 4 to 6 and 13 to 15). GAP contains an SH3 domain, which intervenes between the two SH2 elements and might contribute to binding to receptors. This seems unlikely, because the PLCγ1 SH3 domain, expressed in isolation as a TrpE fusion protein, did not associate with the PDGF-R (Figure 9).

Src-like tyrosine kinases and v-Crk also contain SH2 domains, which may bind activated receptors. Consistent with this prediction, bacterial fusion proteins that contained the SH2 domains of $p60^{src}$ or $P47^{gag\text{-}crk}$ bound PDGF-R in lysates of PDGF-stimulated Rat-2 cells (Fig. 9). $p60^{src}$ is a substrate for the PDGF-R (Ralston and Bishop, Proc. Natl. Acad. Sci. U.S.A. 82, 7845 (1985); Gould and

Hunter, Mol. Cell. Biol. 8, 3345 (1988)), and recent evidence suggests that Scr-like kinases are physically associated with activated PDGF-R in vivo (Kypta et al. Cell 62, 481 (1990)). The data herein imply that this interaction involves the Src SH2 domain. Whether the normal homolog of v-Crk complexes with growth factor receptors in vivo remains to be established.

Example 6

Inhibition of in vitro binding of both PLCγ1 and GAP SH2 domains to the activated PDGF-R in Rat-2 cells that overexpress PLCγ1.

Only a minor fraction of activated PDGF-R complexes with PLCγ1 in vivo. A Rat-2 cell line was genetically modified to overexpress PLCγ1 by tenfold as compared with the endogenous enzyme (Rat-2 PLCγ1). There is a proportionate increase in the amount of PDGF-R precipitated with antibodies to PLCγ1 (anti-PLCγ1) after PDGF stimulation of Rat-2 PLCγ1 cells, in comparison with parental Rat-2 cells. If bacterial PLCγ1 SH2 domains bound to the same site(s) on the PDGF-R as did cellular PLCγ1, then overexpression of PLCγ1 should block binding of bacterial PLCγ1 SH2 domains to activated PDGF-R in vitro. To investigate thisRat-2 cells (Figure 10, lanes 1, 2, 5 and 6) or a Rat-2 cell line that overexpressed PLCγ1 by tenfold (R2-PLCγ; lanes 3, 4, 7, 8) were stimulated with PDGF (lanes 1, 3, and 5-8) or maintained without PDGF (lanes 2 and 4). Cell lysates were mixed with immobilized TrpE-PLC-SH2[N] (lanes 1 to 4), TrpE-PLC-SH2[N + C] (lanes 5 and 7), or TrpE-GAP-SH2[N + 3 + C] (lanes 6 to 8). Samples were washed, separated by gel electrophoresis, and immunoblotted with anti-P.Tyr. Similar results were obtained by blotting with anti-PDGF-R.

When the Rat-2 PLCγ1 cell line was stimulated with PDGF, lysed, and incubated with immobilized PLCγ1-SH2[N] or PLCγ1 SH2[N + C], only one-third as much PDGF-R associated with the bacterial protein, compared with the

parental PDGF-stimulated Rat-2 cells (Figure 10). Binding of TrpE-GAP-SH2 fusion protein to the PDGF-R was also reduced by overexpression of endogenous PLCγ1, suggesting that PLCγ1 and GAP compete for sites on the activated PDGF-R.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for assaying a medium for the presence of a substance that affects an SH2-phosphorylated ligand regulatory system comprising providing an SH2-like domain or a subdomain thereof, and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof to form an SH2-phosphorylated ligand complex. said SH2-like domain or subdomain and/or said phosphorylated ligand being present in a known concentration, and incubating with a test substance which is suspected of affecting an SH2-phosphorylated ligand regulatory system, under conditions which permit the formation of said SH2-phosphorylated ligand complex, and assaying for said SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand.

2. A method for assaying a medium for the presence of an agonist or antagonist substance of an SH2-phosphorylated ligand regulatory system comprising providing an SH2-like domain or a subdomain thereof, and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof to form an SH2-phosphorylated ligand complex, said SH2-like domain or subdomain and/or said phosphorylated ligand being present in a known concentration, and incubating with a suspected agonist or antagonist substance, under conditions which permit the formation of said SH2-phosphorylated ligand complex, and assaying for said SH2-phosphorylated ligand complex, free SH2-like domain or subdomains thereof, or non-complexed phosphorylated ligand.

3. A method as claimed in claim 1, wherein the phosphorylated ligand is a phosphotyrosine or phosphoserine/phosphothreonine polypeptide or peptide.

4. A method as claimed in claim 3, wherein the phosphorylated ligand is an SH2 binding site on a transmembrane receptor with inducible protein-tyrosine kinase activity or a cytoplasmic tyrosine phosphorylated protein.

5. A method as claimed in claim 1, wherein the SH2 domain or subdomain thereof is a sequence which is substantially homologous to an Src homology region 2 (SH2 region), or a subdomain of an SH2 region preferably a conserved region of an SH2 region.

6. A method as claimed in claim 5, wherein the SH2 domain or subdomain thereof is a sequence which is substantially homologous to the SH2 domain shown in Figure 2.

7. A method as claimed in claim 5, wherein the SH2 domain or subdomain thereof is a sequence which is substantially homologous to one or more of the subdomains of the SH2 domain shown in Figure 2.

8. A method as claimed in claim 1, wherein the substance assayed for affects an SH2-phosphorylated ligand regulatory system which regulates transformation pathways.

9. An isolated SH2-phosphorylated ligand complex comprising an SH2-like domain or a subdomain thereof and a phosphorylated ligand which is capable of interacting with said SH2-like domain or a subdomain thereof.

10. A method of using an isolated SH2-like domain or a subdomain thereof to screen for phosphorylated ligands which are active in an SH2-phosphorylated ligand regulatory system.

11. A method of using an isolated SH2-like domain or a subdomain thereof to regulate the interaction of a signalling protein with a related phosphorylated ligand.

12. A pharmaceutical composition comprising an isolated SH2-like domain or a subdomain thereof for use as an agonist or antagonist of the interaction of a signalling protein with a related phosphorylated ligand.

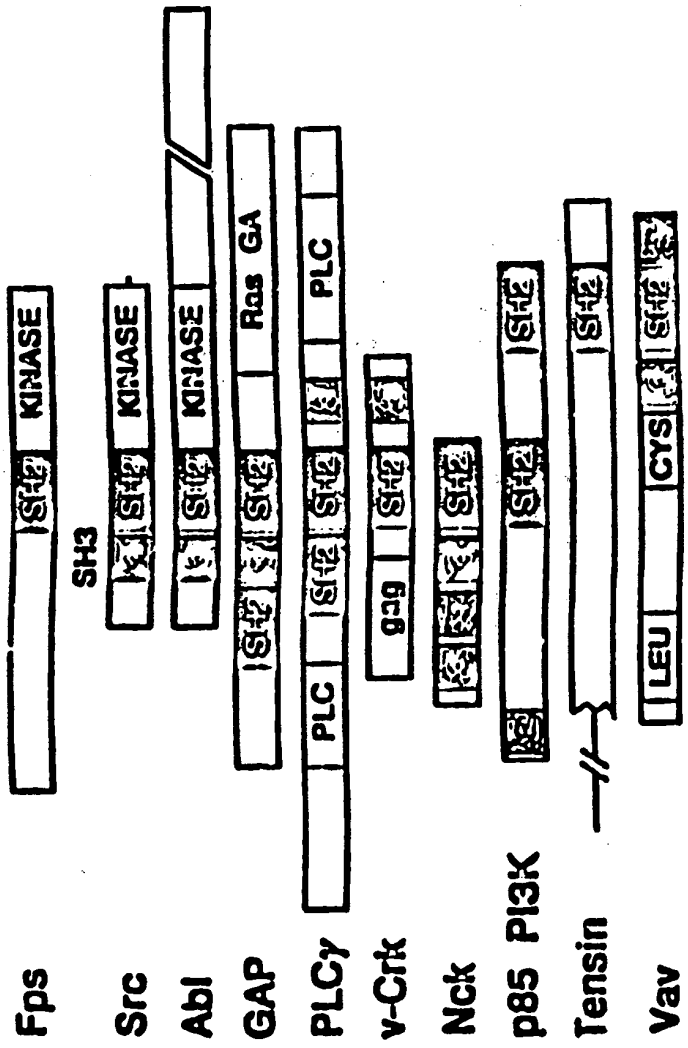

FIGURE 1

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FIGURE 2

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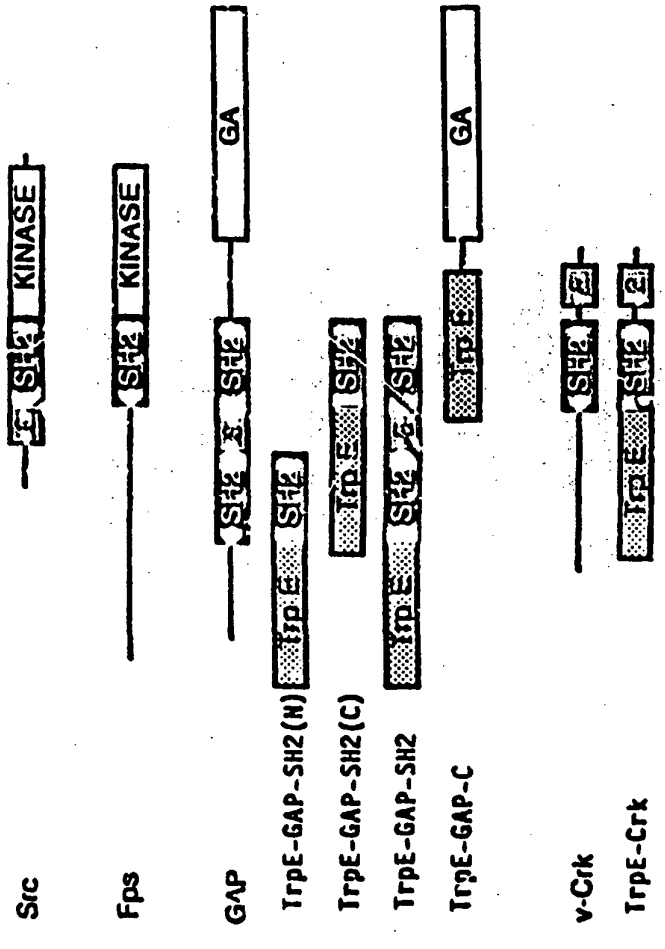

FIGURE 3

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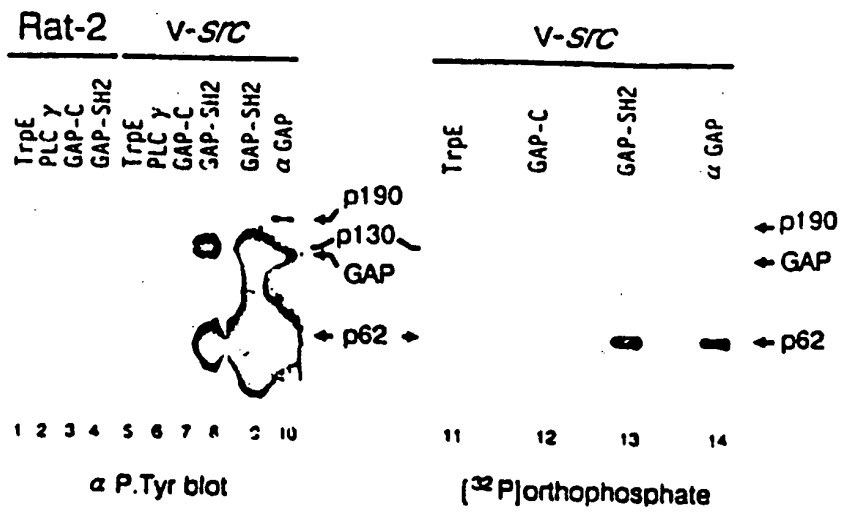

FIGURE 4A

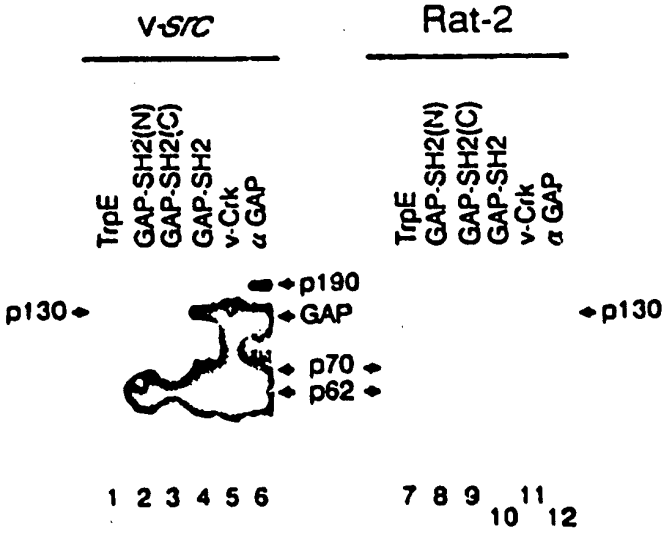

FIGURE 4B

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FIGURE 5A

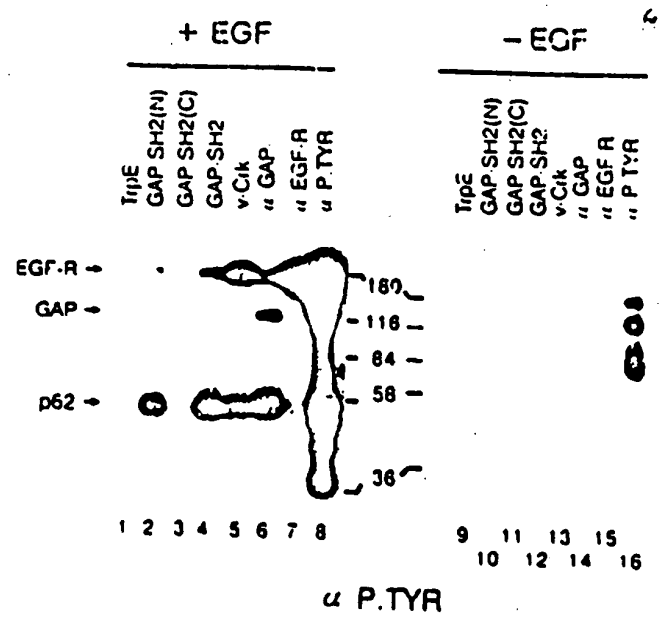
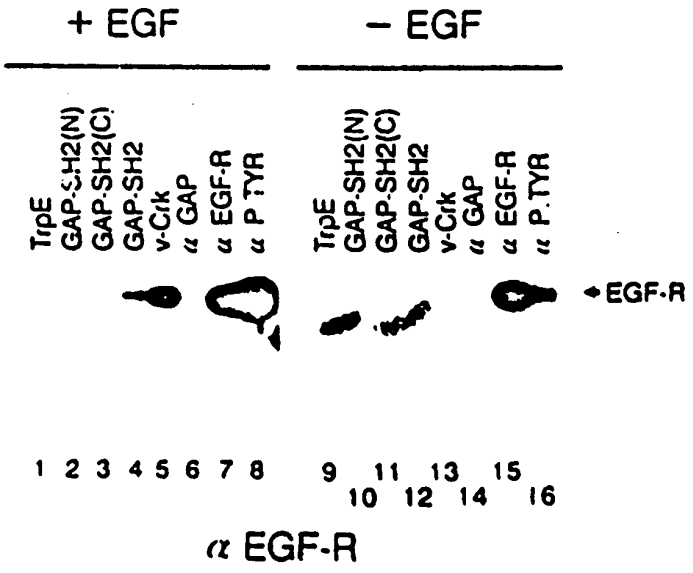


FIGURE 5B



By: Lyon, Brinkin Pat.

FIGURE 6A

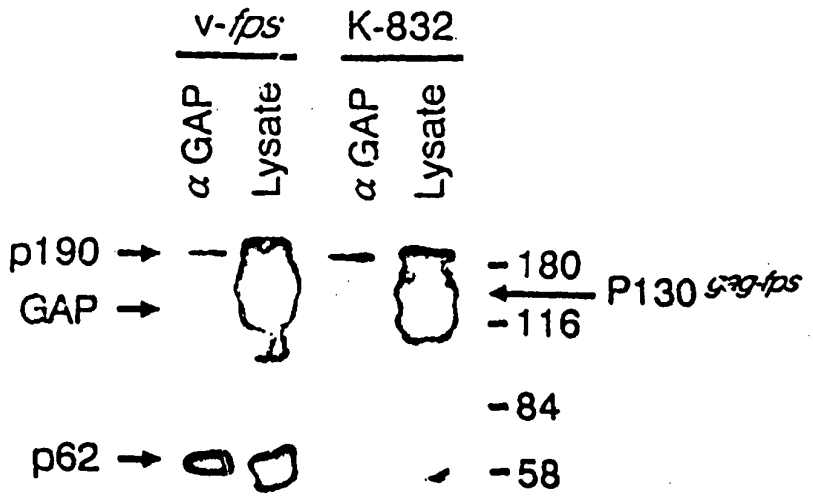
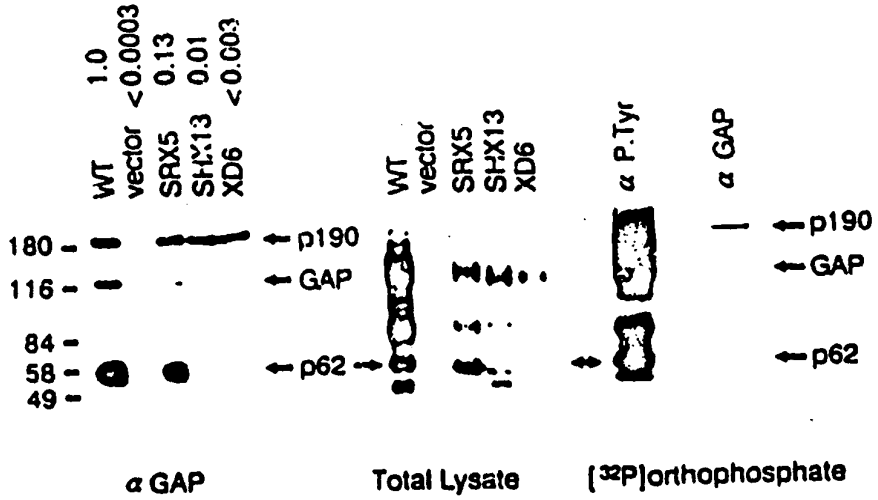


FIGURE 6B



By: Roger Brown & Pan

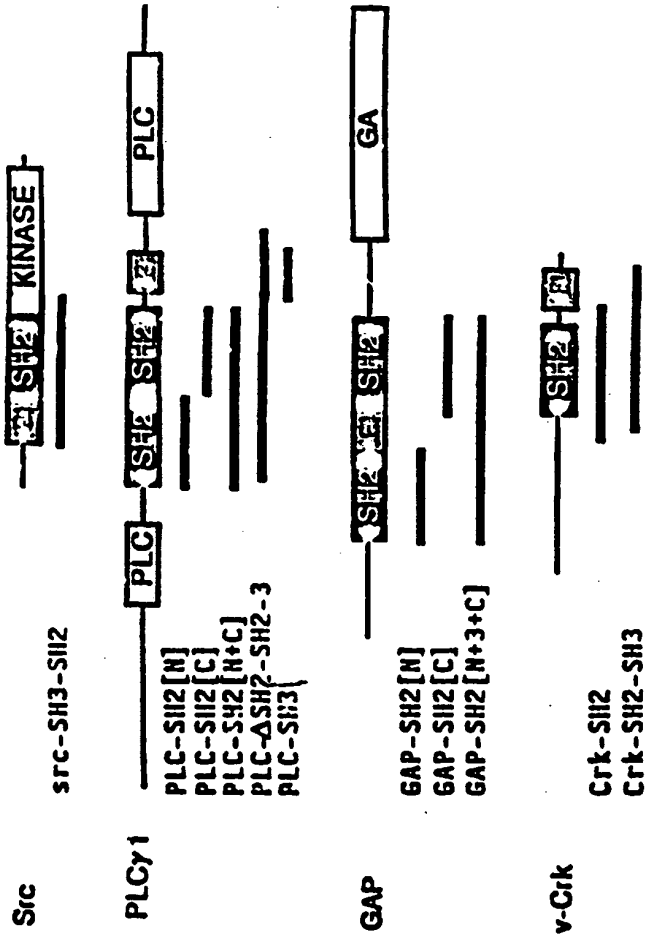


FIGURE 7

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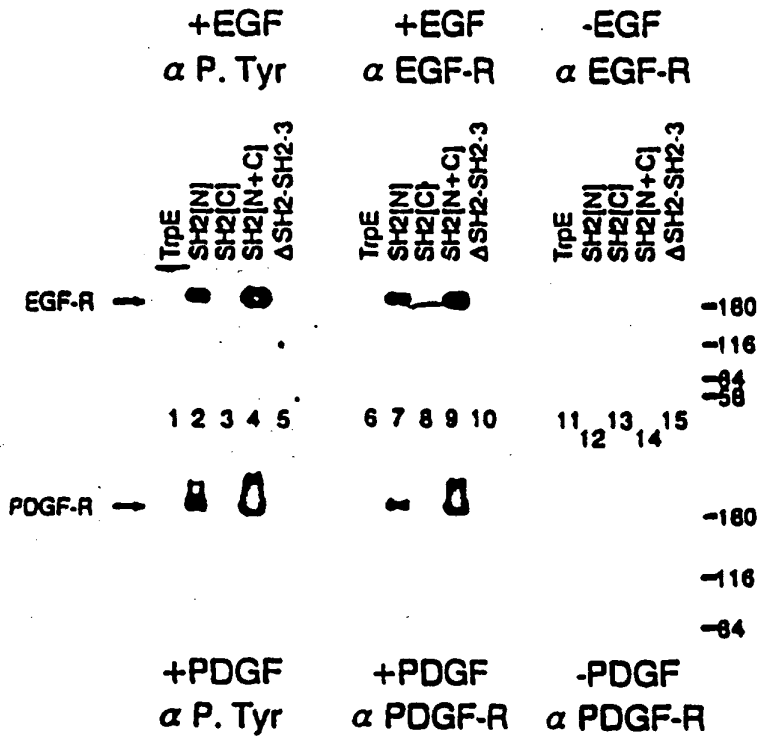

FIGURE 8

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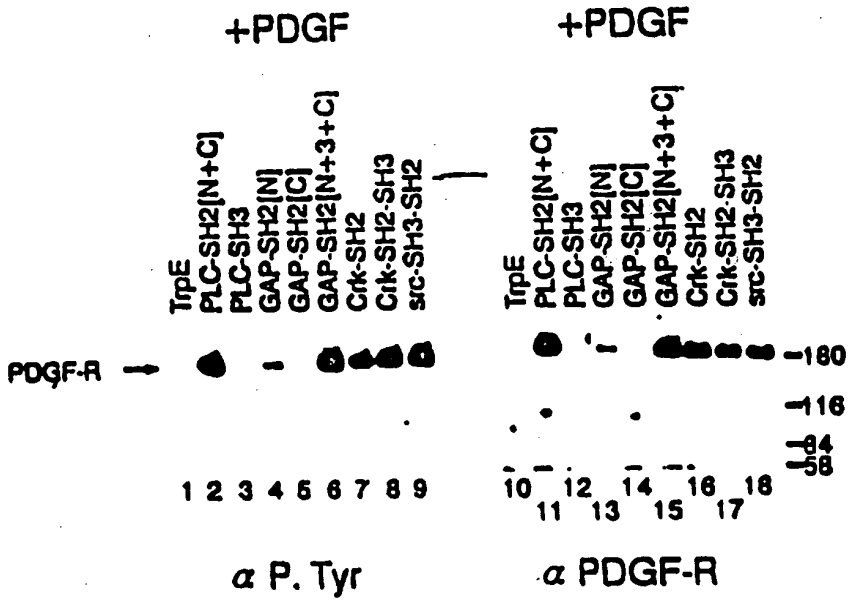

FIGURE 9

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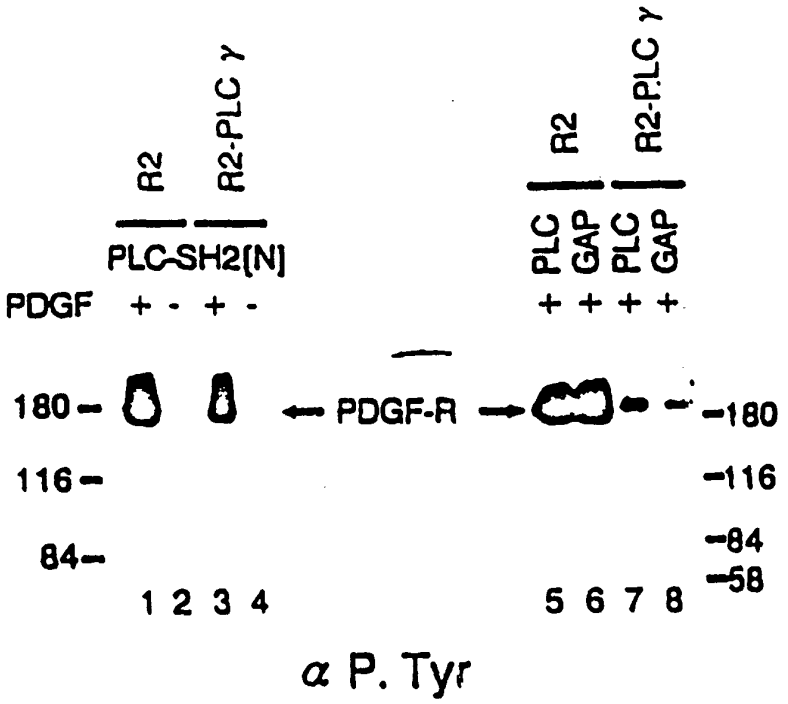

FIGURE 10

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(71) Applicant *(for all designated States except US)*: AFFYMAX TECHNOLOGIES N.V. [NL/NL]; De Ruyderkade 62, Curaçao (AN).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : DOWER, William, J. [US/US]; 761 Partridge Avenue, Menlo Park, CA 94025 (US). CWIRLA, Steven, E. [US/US]; 765 San Antonio Road, #64, Palo Alto, CA 94303 (US). BARRETT, Ronald, W. [US/US]; 562 Carlise Way, Sunnyvale, CA 94087 (US).

(74) Agent: SMITH, William, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).

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(54) Title: PEPTIDE LIBRARY AND SCREENING SYSTEMS

(57) Abstract

Peptide which bind to selected receptors are identified by screening libraries which encode a random or controlled collection of amino acids. Peptides encoded by the libraries are expressed as fusion proteins of bacteriophage coat proteins, and bacteriophage are then screened against the receptors of interest. Peptides having a wide variety of uses, such as therapeutic or diagnostic reagents, may thus be identified without any prior information on the structure of the expected ligand or receptor.

generic ref for screening of peptide library encoded by bacteriophages

no SH3 disclosed

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PEPTIDE LIBRARY AND SCREENING SYSTEMS

Field of the Invention

The present invention relates generally to methods for selecting peptide ligands to receptor molecules of interest and, more particularly, to methods for generating and screening large peptide libraries for peptides with desired binding characteristics.

Background of the Invention

As molecular biology has helped to define regions of proteins that contribute to a particular biological activity, it has become desirable to synthesize short peptides to mimic (or inhibit) those activities. Many of the disadvantages encountered in therapeutic, diagnostic and industrial settings with purified proteins, or those produced by recombinant means, could easily be avoided by short synthetic peptides. For instance, synthetic peptides offer advantages of specificity, convenience of sample or bulk preparation, lower relative cost, high degree of purity, and long shelf-life.

Despite the great promise of synthetic peptides, the technology remains, to a large extent, a laboratory tool. Precise sequence and binding data are not available for most proteins of significant medical, agricultural or industrial interest. Even when the sequence of a protein is known, the process of

identifying short sequences which are responsible for or contribute to a biological activity may be extremely tedious, if not nearly impossible in many instances.

Thus, the ability to generate and efficiently screen very large collections of peptides for desired binding activities would be of enormous interest. It would enable the identification of novel agonists and antagonists for receptors, the isolation of specific inhibitors of enzymes, provide probes for structural and functional analyses of binding sites of many proteins, and ligands for many other compounds employed in a wide variety of applications.

The generation of large numbers of peptide sequences by the cloning and expression of randomly-generated mixtures of oligonucleotides is possible in the appropriate recombinant vectors. See, e.g., Oliphant et al., Gene 44:177-183 (1986). Such a large number of compounds can be produced, however, that methods for efficient physical and genetic selection are required. Without such methods the usefulness of these large peptide libraries in providing ligands of potential interest may be lost. The present invention provides methods for efficient screening and selection from a large peptide library, fulfilling these and other related needs.

Summary of the Invention

The present invention provides novel methods and compositions for identifying peptides which bind to preselected receptor molecules. The peptides find a variety of therapeutic, diagnostic and related uses, e.g., to bind the receptor or an analogue thereof and inhibit or promote its activity.

In one embodiment the invention relates to methods for identifying the peptides which bind to a

preselected receptor. In certain aspects the methods generally comprise constructing a bacteriophage expression vector which comprises an oligonucleotide library of at least about 10^6 members which encode the peptides. The library member is joined in reading frame to the 5' region of a nucleotide sequence encoding an outer structural protein of the bacteriophage. Appropriate host cells are transformed with the expression vectors, generally by electroporation, and the transformed cells cultivated under conditions suitable for expression and assembly of bacteriophage. Using an affinity screening process, bacteriophage library members are contacted with the preselected receptor under conditions conducive to specific peptide-receptor binding, and bacteriophage whose coat proteins have peptides which bind the receptor molecule are selected. The nucleotide sequence which encodes the peptide on the selected phage may then be determined. By repeating the affinity selection process one or more times, the peptides of interest may be enriched. By increasing the stringency of the selection, e.g., by reducing the valency of the peptide-phage interaction towards substantial monovalency, peptides of increasingly higher affinity can be identified.

In another aspect the methods are concerned with expression vectors having the oligonucleotide library members joined in reading frame with a nucleotide sequence to encode a fusion protein, wherein the library member represents the 5' member of the fusion and the 3' member comprises at least a portion of an outer structural protein of the bacteriophage. The first residue of the peptide encoded by the library member may be at the 5'-terminus of the sequence encoding the phage coat protein. In preferred embodiments, where phage proteins are initially expressed as preproteins and then processed by the host cell to a mature protein, the library members are inserted so as to leave the peptide

encoded thereby at the N-terminus of the mature phage protein after processing or a protein substantially homologous thereto.

The invention also concerns host cells transformed with a bacteriophage expression vector having an oligonucleotide library member, joined in reading frame to the 5' region of a nucleotide sequence encoding an outer structural protein of the bacteriophage, wherein the library member encodes a peptide of at least about five to twenty-five amino acids.

Generally, the oligonucleotide library of the invention comprises a variable codon region which encodes for the peptides of interest, and may optionally comprise sequences coding for one or more spacer amino acid residues, such as Gly. The variable region may be encoded by $(NNK)_x$ or $(NNS)_x$, where N is A, C, G or T, K is G or T, S is G or C, and x is from 5 to at least about 8. In certain preferred embodiments the variable region of the oligonucleotide library member encodes a hexapeptide. The variable codon region may also be prepared from a condensation of activated trinucleotides.

Brief Description of the Drawings

Fig. 1 depicts the construction of an oligonucleotide library. (A) The vector fAFF1 contains two non-complementary BstXI sites separated by a 30 bp stuffer fragment. Removal of the BstXI fragment allows oriented ligation of oligonucleotides with the appropriate cohesive ends. (B) The oligonucleotide ON-49 was annealed to two "half-site" fragments to form cohesive termini complementary to BstXI sites 1 and 2 in the vector. The gapped structure, where the single-stranded region comprises the variable hexacodon sequence

and a 2 (gly) codon spacer, was ligated to the vector and electro-transformed into E. coli.

Fig. 2 depicts the amino acid sequences (deduced from DNA sequence) of the N-terminal hexapeptides on pIII of infectious phage randomly selected from the library. Sequences begin at the signal peptidase site. Single letter code for amino acids is A (Ala), C (Cys), D (Asp), E (Glu), F (Phe), G (Gly), H (His), I (Ile), K (Lys), L (Leu), M (Met), N (Asn), P (Pro), Q (Gln), R (Arg), S (Ser), T (Thr), V (Val), W (Trp), Y (Tyr).

Fig. 3 illustrates the composite DNA sequence of the variable region of pools of (A) infectious phage from the library, and (B) phage recovered from 1, 2, or 3 rounds of panning on mAB 3E7. Phage were amplified as tetracycline resistant colonies and DNA from a pool of phage derived from several thousand of these colonies was isolated and sequenced. The area of the sequencing gel corresponding to the cloning site in geneIII is displayed. A sequencing primer was annealed to the phage DNA ~40 bases to the 3' side of the cloning site. The actual readout of the gel is the sequence complementary to the coding strand. For clarity of codon identification, the lanes may be read as C, T, A, G, left to right and 5' to 3', top to bottom, to identify the sequence of the coding (+) strand.

Fig. 4 shows the amino acid sequences (deduced from DNA sequence) of the N-terminal peptides of pIII of 51 phage isolated by three rounds of panning on mAB 3E7.

Fig. 5 illustrates the results of phage sandwich ELISAs for YGGFL- and YAGFAQ-phage with biotinylated monoclonal antibody 3E7 IgG (Fig. 5A) or 3E7 Fab fragments (Fig. 5B) immobilized at maximal density on streptavidin coated wells and labeled polyclonal anti-phage antibodies to detect bound phage.

Fig. 6 illustrates the results of phage sandwich ELISAs which compare the effect of 3E7 Fab

concentration at 5 nM (Fig. 6A) and 50 pM (Fig. 6B) and wash times (minutes) on recoveries of YGGFL- and YAGFAQ-phage.

Fig. 7 shows 3E7 Fab dissociation from phage bearing peptides of known affinity, YGGFL and YGFWGM.

Description of the Preferred Embodiments

Methods and compositions are provided for identifying peptides which bind to receptor molecules of interest. The peptides are produced from oligonucleotide libraries which encode peptides attached to a bacteriophage structural protein. A method of affinity enrichment allows a very large library of peptides to be screened and the phage carrying the desired peptide(s) selected. The nucleic acid may then be isolated from the phage and the variable region of the oligonucleotide library member sequenced, such that the amino acid sequence of the desired peptide is deduced therefrom. Using these methods a peptide identified as having a binding affinity for the desired molecule may then be synthesized in bulk by conventional means.

By identifying the peptide de novo one need not know the sequence or structure of the receptor molecule or the sequence of its natural binding partner. Indeed, for many "receptor" molecules a binding partner has not yet been identified. A significant advantage of the present invention is that no prior information regarding an expected ligand structure is required to isolate peptide ligands of interest. The peptide identified will thus have biological activity, which is meant to include at least specific binding affinity for a selected receptor molecule, and in some instances will further include the ability to block the binding of other compounds, to stimulate or inhibit metabolic pathways, to

act as a signal or messenger, to stimulate or inhibit cellular activity, and the like.

The number of possible receptor molecules for which peptide ligands may be identified by means of the present invention is virtually unlimited. For example, the receptor molecule may be an antibody (or a binding portion thereof). The antigen to which the antibody binds may be known and perhaps even sequenced, in which case the invention may be used to map epitopes of the antigen. If the antigen is unknown, such as with certain autoimmune diseases, for example, sera or other fluids from patients with the disease can be used in the present methods to identify peptides, and consequently the antigen which elicits the autoimmune response. It is also possible using these methods to tailor a peptide to fit a particular individual's disease. Once a peptide has been identified it may itself serve as, or provide the basis for, the development of a vaccine, a therapeutic agent, a diagnostic reagent, etc.

The present invention can identify peptide ligands for a wide variety of substances in addition to antibodies. These include, by way of example and not limitation, growth factors, hormones, enzymes, interferons, interleukins, intracellular and intercellular messengers, lectins, cellular adhesion molecules and the like, as well as the ligands for the corresponding receptors of the aforementioned molecules. It will be recognized that peptide ligands may also be identified by the present invention for molecules which are not peptides or proteins, e.g., carbohydrates, non-protein organic compounds, metals, etc. Thus, although antibodies are widely available and conveniently manipulated, they are merely representative of receptor molecules for which peptide ligands can be identified by means of the present invention.

An oligonucleotide library, prepared according to the criteria as described herein, is inserted in an

appropriate vector encoding a bacteriophage structural protein, preferably an accessible phage protein, such as a bacteriophage coat protein. Although one skilled in the art will appreciate that a variety of bacteriophage may be employed in the present invention, in preferred embodiments the vector is, or is derived from, a filamentous bacteriophage, such as, for example, f1, fd, Pf1, M13, etc. In a more preferred embodiment the filamentous phage is fd, and contains a selectable marker such as tetracycline (e.g., "fd-tet"). The fd-tet vector has been extensively described in the literature. See, for example, Zacher et al., Gene 9:127-140 (1980), Smith et al., Science 228:1315-1317 (1985) and Parmley and Smith, Gene 73:305-318 (1988), each incorporated by reference herein.

The phage vector is chosen to contain or is constructed to contain a cloning site located in the 5' region of the gene encoding the bacteriophage structural protein, so that the peptide is accessible to receptors in an affinity selection and enrichment procedure as described below. As the structural phage protein is preferably a coat protein, in phage fd the preferred coat protein is pIII. Each filamentous fd phage is known to have up to four or five copies of the pIII protein.

An appropriate vector allows oriented cloning of the oligonucleotide sequences which encode the peptide so that the peptide is expressed at or within a distance of about 100 amino acid residues of the N-terminus of the mature coat protein. The coat protein is typically expressed as a preprotein, having a leader sequence. Thus, desirably the oligonucleotide library is inserted so that the N-terminus of the processed bacteriophage outer protein is the first residue of the peptide, i.e., between the 3'-terminus of the sequence encoding the leader protein and the 5'-terminus of the sequence encoding the mature protein or a portion of the 5' terminus.

The library is constructed by cloning an oligonucleotide which contains the variable region of library members (and any spacers, framework determinants, etc. as discussed below) into the selected cloning site. Using known recombinant DNA techniques (see generally, Sambrooke et al., Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, incorporated by reference herein), an oligonucleotide may be constructed which, inter alia, removes unwanted restriction sites and adds desired ones, reconstructs the correct portions of any sequences which have been removed (such as a correct signal peptidase site, for example), inserts the spacer conserved or framework residues, if any, and corrects the translation frame (if necessary) to produce active, infective phage. The central portion of the oligonucleotide will generally contain one or more of the variable region domain(s) and the spacer or framework residues. The sequences are ultimately expressed as peptides (with or without spacer or framework residues) fused to or in the N-terminus of the mature coat protein on the outer, accessible surface of the assembled bacteriophage particles.

The variable region domain of the oligonucleotide comprises the source of the library. The size of the library will vary according to the number of variable codons, and hence the size of the peptides, which are desired. Generally the library will be at least about 10^6 members, usually at least 10^7, and typically 10^8 or more members. To generate the collection of oligonucleotides which forms a series of codons encoding a random collection of amino acids and which is ultimately cloned into the vector, a codon motif is used, such as $(NNK)_x$, where N may be A, C, G, or T (nominally equimolar), K is G or T (nominally equimolar), and x is typically up to about 5, 6, 7, or 8 or more, thereby producing libraries of penta-, hexa-, hepta-, and octa-peptides or more. The third position may also be G or C,

designated "S". Thus, NNK or NNS (i) code for all the amino acids, (ii) code for only one stop codon, and (iii) reduce the range of codon bias from 6:1 to 3:1. It should be understood that with longer peptides the size of the library which is generated may become a constraint in the cloning process and thus the larger libraries can be sampled, as described hereinbelow. The expression of peptides from randomly generated mixtures of oligonucleotides in appropriate recombinant vectors is discussed in Oliphant et al., Gene 44:177-183 (1986), incorporated herein by reference.

An exemplified codon motif $(NNK)_6$ produces 32 codons, one for each of 12 amino acids, two for each of five amino acids, three for each of three amino acids and one (amber) stop codon. Although this motif produces a codon distribution as equitable as available with standard methods of oligonucleotide synthesis, it results in a bias against peptides containing one-codon residues. For example, a complete collection of hexacodons contains one sequence encoding each peptide made up of only one-codon amino acids, but contains 729 (3^6) sequences encoding each peptide with only three-codon amino acids.

An alternative approach to minimize the bias against one-codon residues involves the synthesis of 20 activated tri-nucleotides, each representing the codon for one of the 20 genetically encoded amino acids. These are synthesized by conventional means, removed from the support but maintaining the base and 5-HO-protecting groups, and activating by the addition of 3'O-phosphoramidite (and phosphate protection with b-cyanoethyl groups) by the method used for the activation of mononucleosides, as generally described in McBride and Caruthers, Tetrahedron Letters 22:245 (1983), which is incorporated by reference herein. Degenerate "oligocodons" are prepared using these trimers as building blocks. The trimers are mixed at the desired molar ratios and installed in the synthesizer. The

ratios will usually be approximately equimolar, but may be a controlled unequal ratio to obtain the over- to under-representation of certain amino acids coded for by the degenerate oligonucleotide collection. The condensation of the trimers to form the oligocodons is done essentially as described for conventional synthesis employing activated mononucleosides as building blocks. See generally, Atkinson and Smith, Oligonucleotide Synthesis, M.J. Gait, ed. p35-82 (1984). Thus, this procedure generates a population of oligonucleotides for cloning that is capable of encoding an equal distribution (or a controlled unequal distribution) of the possible peptide sequences. This approach may be especially useful in generating longer peptide sequences, since the range of bias produced by the $(NNK)_6$ motif increases by three-fold with each additional amino acid residue.

When the codon motif is $(NNK)_x$, as defined above, and when x equals 8, there are 2.6×10^{10} possible octa-peptides. A library containing most of the octa-peptides may be difficult to produce. Thus, a sampling of the octa-peptides may be accomplished by constructing a subset library using of about .1%, and up to as much as 1%, 5% or 10% of the possible sequences, which subset of recombinant bacteriophage particles is then screened. As the library size increases, smaller percentages are acceptable. If desired, to extend the diversity of a subset library the recovered phage subset may be subjected to mutagenesis and then subjected to subsequent rounds of screening. This mutagenesis step may be accomplished in two general ways: the variable region of the recovered phage may be mutagenized, or additional variable amino acids may be added to the regions adjoining the initial variable sequences.

A variety of techniques can be used in the present invention to diversify a peptide library or to diversify around peptides found in early rounds of panning to have sufficient binding activity. In one

approach, the positive phage (those identified in an early round of panning) are sequenced to determine the identity of the active peptides. Oligonucleotides are then synthesized based on these peptide sequences, employing a low level of all bases incorporated at each step to produce slight variations of the primary oligonucleotide sequences. This mixture of (slightly) degenerate oligonucleotides is then cloned into the affinity phage as described herein. This method produces systematic, controlled variations of the starting peptide sequences. It requires, however, that individual positive phage be sequenced before mutagenesis, and thus is useful for expanding the diversity of small numbers of recovered phage.

Another technique for diversifying around the recognition kernal of the selected phage-peptide involves the subtle misincorporation of nucleotide changes in the peptide through the use of the polymerase chain reaction (PCR) under low fidelity conditions. A protocol of Leung at al., Technique 1:11-15 (1989) alters the ratios of nucleotides and the addition of manganese ions to produce a 2% mutation frequency. Yet another approach for diversifying the selected phage involves the mutagenesis of a pool, or subset, of recovered phage. Phage recovered from panning are pooled and single stranded DNA is isolated. The DNA is mutagenized by treatment with, e.g., nitrous acid, formic acid, or hydrazine. These treatments produce a variety of damage in the DNA. The damaged DNA is then copied with reverse transcriptase which misincorporates bases when it encounters a site of damage. The segment containing the sequence encoding the variable peptide is then isolated by cutting with restriction nuclease(s) specific for sites flanking the variable region. This mutagenized segment is then recloned into undamaged vector DNA in a manner similar to that described herein. The DNA is transformed into cells and a secondary library is constructed as described. The

general mutagenesis method is described in detail in Myers, et al., Nucl. Acids Res. 13:3131-3145 (1985), Myers et al., Science 229:242-246 (1985), and Myers, Current Protocols in Molecular Biology Vol I, 8.3.1 - 8.3.6, F. Ausebel, et al., eds, J. Wiley and Sons, New York (1989), each of which are incorporated herein by reference.

In the second general approach, that of adding additional amino acids to a peptide or peptides found to be active, a variety of methods are available. In one, the sequences of peptides selected in early panning are determined individually and new oligonucleotides, incorporating the determined sequence and an adjoining degenerate sequence, are synthesized. These are then cloned to produce a secondary library.

In another approach which adds a second variable region to a pool of peptide-bearing phage, a restriction site is installed next to the primary variable region. Preferably, the enzyme should cut outside of its recognition sequence, such as BspMI which cuts leaving a four base 5' overhang, four bases to the 3' side of the recognition site. Thus, the recognition site may be placed four bases from the primary degenerate region. To insert a second variable region, the pool of phage DNA is digested and blunt-ended by filling in the overhang with Klenow fragment. Double-stranded, blunt-ended, degenerately synthesized oligonucleotide is then ligated into this site to produce a second variable region juxtaposed to the primary variable region. This secondary library is then amplified and screened as before.

While in some instances it may be appropriate to synthesize peptides having contiguous variable regions to bind certain receptors, in other cases it may be desirable to provide peptides having two or more regions of diversity separated by spacer residues. For example, the variable regions may be separated by spacers which

allow the diversity domains of the peptides to be presented to the receptor in different ways. The distance between variable regions may be as little as one residue, sometimes five to ten and up to about 100 residues. For probing a large binding site the variable regions may be separated by a spacer of residues of 20 to 30 amino acids. The number of spacer residues when present will preferably be at least two, typically at least three or more, and often will be less than ten, more often less than eight residues.

Thus, an oligonucleotide library having variable domains separated by spacers can be represented by the formula:

$$(NNK)_y-(abc)_n-(NNK)_z,$$

where N and K are as defined previously (note that S as defined previously may be substituted for K), and y + z is equal to about 5, 6, 7, 8, or more, a, b and c represent the same or different nucleotides comprising a codon encoding spacer amino acids, n is up to about 20 to 30 amino acids or more.

The spacer residues may be somewhat flexible, comprising oligo-glycine, for example, to provide the diversity domains of the library with the ability to interact with sites in a large binding site relatively unconstrained by attachment to the phage protein. Rigid spacers, such as, e.g., oligo-proline, may also be inserted separately or in combination with other spacers, including Gly. It may be desired to have the variable domains close to one another and use a spacer to orient the variable domain with respect to each other, such as by employing a turn between the two sequences, as might be provided by a spacer of the sequence Gly-Pro-Gly, for example. To add stability to such a turn, it may be desirable or necessary to add Cys residues at either or both ends of each variable region. The Cys residues would then form disulfide bridges to hold the variable regions together in a loop, and in this fashion may also

serve to mimic a cyclic peptide. Of course, those skilled in the art will appreciate that various other types of covalent linkages for cyclization may also be accomplished.

The spacer residues described above can also be situated on either or both ends of the variable nucleotide region. For instance, a cyclic peptide may be accomplished without an intervening spacer, by having a Cys residue on both ends of the peptide. As above, flexible spacers, e.g., oligo-glycine, may facilitate interaction of the peptide with the selected receptors. Alternatively, rigid spacers may allow the peptide to be presented as if on the end of a rigid arm, where the number of residues, e.g., Pro, determines not only the length of the arm but also the direction for the arm in which the peptide is oriented. Hydrophilic spacers, made up of charged and/or uncharged hydrophilic amino acids, (e.g., Thr, His, Asn, Gln, Arg, Glu, Asp, Met, Lys, etc.), or hydrophobic spacers of hydrophobic amino acids (e.g., Phe, Leu, Ile, Gly, Val, Ala, etc.) may be used to present the peptides to binding sites with a variety of local environments.

Unless modified during or after synthesis by the translation machinery, recombinant peptide libraries consist of sequences of the 20 normal L-amino acids. While the available structural diversity for such a library is large, additional diversity can be introduced by a variety of means, such as chemical modifications of the amino acids.

For example, as one source of added diversity a peptide library of the invention can have its carboxy terminal amidated. Carboxy terminal amidation is necessary to the activity of many naturally occurring bioactive peptides. This modification occurs <u>in vivo</u> through cleavage of the N-C bond of a carboxy terminal Gly residue in a two-step reaction catalyzed by the enzymes peptidylglycine alpha-amidation monooxygenase

(PAM) and hydroxyglycine aminotransferase (HGAT). See, Eipper et al., J. Biol. Chem. 266:7827-7833 (1991); Mizuno et al., Biochem. Biophys. Res. Comm. 137(3): 984-991 (1986); Murthy et al., J. Biol. Chem. 261(4): 1815-1822 (1986); Katopodis et al., Biochemistry 29:6115-6120 (1990); and Young and Tamburini, J. Am. Chem. Soc. 111:1933-1934 (1989), each of which are incorporated herein by reference.

Carboxy terminal amidation can be made to a peptide library of the invention which has the variable region exposed at the carboxy terminus. Amidation can be performed by treatment with enzymes, such as PAM and HGAT, in vivo or in vitro, and under conditions conducive to maintaining the structural integrity of the bioactive peptide. In a random peptide library of the present invention, amidation will occur on a library subset, i.e., those peptides having a carboxy terminal Gly. A library of peptides designed for amidation can be constructed by introducing a Gly codon at the end of the variable region domain of the library. After amidation, an enriched library serves as a particularly efficient source of ligands for receptors that preferentially bind amidated peptides.

Many of the C-terminus amidated bioactive peptides are processed from larger pro-hormones, where the amidated peptide is flanked at its C-terminus by the sequence -Gly-Lys-Arg-X... (where X is any amino acid). In the present invention, oligonucleotides encoding the sequence -Gly-Lys-Arg-X-Stop are placed at the 3' end of the variable oligonucleotide region. When expressed, the Gly-Lys-Arg-X is removed by in vivo or in vitro enzymatic treatment and the peptide library is carboxy terminal amidated as described above.

Another means to add to the library diversity through carboxy terminal amidation involves the use of proteins that typically have an exposed C terminus, i.e., a protein that crosses a membrane with its carboxy

terminus exposed on the extracellular side of the membrane. In this embodiment the variable oligonucleotides region, having a stop codon in the last position, is inserted in the 3' end of a sequence which encodes C terminus exposed protein, or at least a portion of the protein that is responsible for the C-terminus out orientation. The transferrin receptor protein is an example of one such protein. This receptor has been cloned and sequenced, as reported in McClelland et al., Cell 39:267-274 (1984), incorporated herein by reference. An internal transmembrane segment of the transferrin receptor serves to orient the protein with its carboxy terminus out. When the cDNA is expressed, typically in eucaryotic cells, the random peptides are located extracellularly, having their amino terminus fused to the transferrin receptor and with a free carboxy terminus.

For carboxy terminal peptide libraries, a COS cell expression cloning system can also be used and may be preferred in some circumstances. COS cells are transfected with a variable nucleotide library contained in an expression plasmid that replicates and produces mRNA extrachromosomally when transfected into COS cells. Transfected cells bearing the random peptides are selected on immobilized ligand or cells which bear a binding protein, and the plasmid is isolated (rescued) from the selected cells. The plasmid is then amplified and used to transfect COS cells for a second round of screening. Because the random oligonucleotides are inserted directly into the expression plasmid, much larger libraries (i.e., total number of novel peptides) are constructed. Of course, for each round of panning the plasmid needs to be rescued from the COS cells, transfected into bacteria for amplification, re-isolated and transfected back into COS cells.

Other expression systems for carboxy terminal amidation of peptides of the invention can also be used. For example, the variable oligonucleotide sequences are

inserted into the 3' end of, e.g., the transferrin receptor cDNA contained in a baculovirus transfer vector. Viral DNA and transfer vector are co-transfected into insect cells (e.g., Sf9 cells) which are used to propagate the virus in culture. When transferrin receptor is expressed, cells harboring recombinant virus, i.e., those producing the transferrin receptor/variable peptide fusion protein, are selected using an anti-transferrin receptor monoclonal antibody linked to a particle such as magnetic microspheres or other substance to facilitate separation. The selected cells are further propagated, allowed to lyse and release the library of recombinant extracellular budded virus into the media.

The library of recombinant virus is amplified (e.g., in Sf9 cells), and aliquots of the library stored. Sf9 cells are then infected with the library of recombinant virus and panned on immobilized target receptor, where the panning is timed to occur with transferrin receptor expression. The selected cells are allowed to grow and lyse, and the supernatant used to infect new Sf9 cells, resulting in amplification of virus that encodes peptides binding to the target receptor. After several rounds of panning and amplification, single viruses are cloned by a Sf9 cell plaque assay as described in Summers and Smith, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experiment Station Bulletin No. 1555 (1988), incorporated herein by reference. DNA in the variable oligonucleotide insert region is then sequenced to determine the peptides which bind to the target receptor.

An advantage of the baculovirus system for peptide library screening is that expression of the transferrin receptor/random peptide fusion protein is very high (>1 millions receptors per cell). A high expression level increases the likelihood of successful panning based on stoichiometry and/or contributes to

polyvalent interactions with an immobilized target receptor. Another advantage of the baculovirus system is that, similar to the peptide on phage method, infectivity is exploited to amplify virus which is selected by the panning procedure. During the series of pannings, the DNA does not need to be isolated and used for subsequent transfections of cells.

Other expression systems can be employed in the present invention. As eucaryotic signal sequences are operable in yeast and bacteria, proteins with a carboxy terminus out orientation, such as the transferrin receptor, can be appropriately expressed and oriented in yeast or bacteria. The use of yeast or bacteria allows large libraries and avoids potential problems associated with amplification.

Other modifications found in naturally occurring peptides and proteins can be introduced into the libraries to provide additional diversity and to contribute to a desired biological activity. For example, the variable region library can be provided with codons which code for amino acid residues involved in phosphorylation, glycosylation, sulfation, isoprenylation (or the addition of other lipids), etc. Modifications not catalyzed by naturally occurring enzymes can be introduced by chemical means (under relatively mild conditions) or through the action of, e.g., catalytic antibodies and the like. In most cases, an efficient strategy for library construction involves specifying the enzyme (or chemical) substrate recognition site within or adjacent to the variable nucleotide region of the library so that most members of the library are modified. The substrate recognition site added could be simply a single residue (e.g., serine for phosphorylation) or a complex consensus sequence, as desired.

Conformational constraints, or scaffolding, can also be introduced into the structure of the peptide libraries. A number of motifs from known protein and

peptide structures can be adapted for this purpose. The method involves introducing nucleotide sequences that code for conserved structural residues into or adjacent to the variable nucleotide region so as to contribute to the desired peptide structure. Positions nonessential to the structure are allowed to vary.

A degenerate peptide library as described herein can incorporate the conserved frameworks to produce and/or identify members of families of bioactive peptides or their binding receptor elements. Several families of bioactive peptides are related by a secondary structure that results in a conserved "framework," which in some cases is a pair of cysteines that flank a string of variable residues. This results in the display of the variable residues in a loop closed by a disulfide bond, as discussed above.

In some cases a more complex framework is shared among members of a peptide family which contributes to the bioactivity of the peptides. An example of this class is the conotoxins, peptide toxins of 10 to 30 amino acids produced by venomous molluscs known as predatory cone snails. The conotoxin peptides generally possess a high density of disulfide cross-linking. Of those that are highly cross-linked, most belong to two groups, mu and omega, that have conserved primary frameworks as follows:

mu	CC.....C.....C.....CC; and
omega	C.....C.....CC.....C.....C

The number of residues flanked by each pair of C's varies from 2 to 6 in the peptides reported to date. The side chains of the residues which flank the Cys residues are apparently not conserved in peptides with different specificity, as in peptides from different species with similar or identical specificities. Thus, the conotoxins have exploited a conserved, densely cross-linked motif as a framework for hypervariable regions to produce a huge

array of peptides with many different pharmacological effects.

The mu and omega classes (with 6 C's) have 15 possible combinations of disulfide bonds. Usually only one of these conformations is the active ("correct") form. The correct folding of the peptides may be directed by a conserved 40 residue peptide that is cleaved from the N-terminus of the conopeptide to produce the small, mature bioactive peptides that appear in the venom.

With 2 to 6 variable residues between each pair of C's, there are 125 (5^3) possible framework arrangements for the mu class (2,2,2, to 6,6,6), and 625 (5^4) possible for the omega (2,2,2,2 to 6,6,6,6). Randomizing the identity of the residues within each framework produces 10^{10} to $>10^{30}$ peptides. "Cono-like" peptide libraries are constructed having a conserved disulfide framework, varied numbers of residues in each hypervariable region, and varied identity of those residues. Thus, a sequence for the structural framework for use in the present invention comprises Cys-Cys-Y-Cys-Y-Cys-Cys, or Cys-Y-Cys-Y-Cys-Cys-Y-Cys-Y-Cys, wherein Y is $(NNK)_x$ or $(NNS)_x$, and where N is A, C, G or T, K is G or T, S is G or C, and x is from 2 to 6.

Other changes can be introduced to provide residues that contribute to the peptide structure, around which the variable amino acids are encoded by the library members. For example, these residues can provide for α-helices, a helix-turn-helix structure, four helix bundles, etc., as described.

Another exemplary scaffold structure takes advantage of metal ion binding to conformationally constrain peptide structures. Properly spaced invariant metal ligands (cysteines and histidines) for certain divalent cations (e.g., zinc, cobalt, nickel, cadmium, etc.) can be specifically incorporated into the peptide libraries. Depending on the particular motif specified

these can result (in the case of zinc coordination, for example) in zinc loops, zinc fingers, zinc twists, or zinc clusters, as generally described in Berg (J. Biol. Chem. 265:6513-6516 (1990)), Green et al. (Proc. Natl. Acad. Sci. USA, 86:4047-4051 (1989)), Parraga et al. (Science 241:1489-1492 (1988)), Regan et al. (Biochem., 29:10878-10883 (1990)), and Vallee et al. (Proc. Natl. Acad. Sci. USA, 88:999-1003 (1991)), each incorporated herein by reference. Other DNA binding peptides, such as those which correspond to the transcriptional transactivators referred to as leucine zippers, can also be used as a framework, where leucine residues are repeated every seven residues in the motifs, and the region is adjacent to an alpha helical region rich in lysines and arginines and characterized by a conserved helical face and a variable helical face.

Other specialized forms of structural constraints can also be used in the present invention. For example, certain serine proteases are inhibited by small proteins of conserved structure (e.g., pancreatic trypsin inhibitor). This conserved framework can incorporate degenerate regions as described herein to generate libraries for screening for novel protease inhibitors.

In another aspect related to frameworks for a peptide library, information from the structure of known ligands can be used to find new peptide ligands having features modified from those of the known ligand. In this embodiment, fragments of a gene encoding a known ligand, prepared by, e.g., limited DNAse digestion into pieces of 20 to 100 base pairs, are subcloned into a variable nucleotide region system as described herein either singly or in random combinations of several fragments. The fragment library is then screened in accordance with the procedures herein for binding to the receptor to identify small peptides capable of binding to the receptor and having characteristics which differ as

desired from the parental peptide ligand. This is useful for screening for any receptor-ligand interaction where one or both members are encoded by a gene, e.g., growth factors, hormones, cytokines and the like, such as insulin, interleukins, insulin-like growth factor, etc.

The peptide-phage libraries of the present invention can also be used to determine the site specificity of enzymes that modify proteins, e.g., the cleavage specificity of a protease. For example, factor X_a cleaves after the sequence Ile-Glu-Gly-Arg. A library of variable region codons as described herein is constructed having the structure: signal sequence--variable region--Tyr-Gly-Gly-Phe-Leu--pIII. Phage from the library are then exposed to factor X_a and then panned on an antibody (e.g., 3E7), which is specific for N-terminally exposed Tyr-Gly-Gly-Phe-Leu. A pre-cleavage panning step with 3E7 can be employed to eliminate clones cleaved by E. coli proteases. Only members of the library with random sequences compatible with cleavage with factor X_a are isolated after panning, which sequences mimic the Ile-Glu-Gly-Arg site.

Another approach to protease substrate identification involves placing the variable region between the carrier protein and a reporter sequence that is used to immobilize the complex (e.g., Tyr-Gly-Gly-Phe-Leu). Libraries are immobilized using a receptor that binds the reporter sequence (e.g., 3E7 antibody). Phage clones having sequences compatible with cleavage are released by treatment with the desired protease.

Some peptides, because of their size and/or sequence, may cause severe defects in the infectivity of their carrier phage. This causes a loss of phage from the population during reinfection and amplification following each cycle of panning. To minimize problems associated with defective infectivity, DNA prepared from the eluted phage is transformed into appropriate host cells, such as, e.g., E. coli, preferably by

electroporation, as described in, for example, Dower et al., Nucl. Acids Res. 16:6127-6145 (1988), incorporated herein by reference, or by well known chemical means. The cells are cultivated for a period of time sufficient for marker expression, and selection is applied as typically done for DNA transformation. The colonies are amplified, and phage harvested for affinity enrichment as described below. Phage identified in the affinity enrichment can be re-amplified in additional rounds of propagation by infection into appropriate hosts.

The successful transformants are typically selected by growth in a selective medium or under selective conditions, e.g., an appropriate antibiotic, which, in the case of the fd-tet vector, is preferably tetracycline. This may be done on solid or in liquid growth medium. For growth on solid medium, the cells are grown at a high density (~10^8 to 10^9 tfs per m^2) on a large surface of, for example, L-agar containing the selective antibiotic to form essentially a confluent lawn. The cells and extruded phage are scraped from the surface and phage are prepared for first round of panning essentially as described by Parmley and Smith, Gene 73:305-318 (1988). For growth in liquid culture, cells may be grown in L-broth and antibiotic through about 10 or more doublings. The phage are harvested by standard procedures (see Sambrooke et al., Molecular Cloning, 2nd ed. (1989), supra, for preparation of M13 phage) as described below. Growth in liquid culture may be more convenient because of the size of the libraries, while growth on solid media likely provides less chance of bias during the amplification process.

For affinity enrichment of desired clones, generally about 10^3 to 10^4 library equivalents (a library equivalent is one of each recombinant; 10^4 equivalents of a library of 10^9 members is $10^9 \times 10^4 = 10^{13}$ phage), but typically at least 10^2 library equivalents but up to about 10^5 to 10^6, are incubated with a receptor (or portion

thereof) to which the desired peptide is sought. The receptor is in one of several forms appropriate for affinity enrichment schemes. In one example the receptor is immobilized on a surface or particle, and the library of phage bearing peptide is then panned on the immobilized receptor generally according to the procedure described below.

A second example of receptor presentation is receptor attached to a recognizable ligand (which may be attached via a tether). A specific example of such a ligand is biotin. The receptor, so modified, is incubated with the library of phage and binding occurs with both reactants in solution. The resulting complexes are then bound to streptavidin (or avidin) through the biotin moiety. The streptavidin may be immobilized on a surface such as a plastic plate or on particles, in which case the complexes (phage/peptide/receptor/biotin/streptavidin) are physically retained; or the streptavidin may be labelled, with a fluorophore, for example, to tag the active phage/peptide for detection and/or isolation by sorting procedures, e.g., on a fluorescence-activated cell sorter.

Phage which express peptides without the desired specificity are removed by washing. The degree and stringency of washing required will be determined for each receptor/peptide of interest. A certain degree of control can be exerted over the binding characteristics of the peptides recovered by adjusting the conditions of the binding incubation and the subsequent washing. The temperature, pH, ionic strength, divalent cation concentration, and the volume and duration of the washing will select for peptides within particular ranges of affinity for the receptor. Selection based on slow dissociation rate, which is usually predictive of high affinity, is the most practical route. This may be done either by continued incubation in the presence of a

saturating amount of free ligand, or by increasing the volume, number, and length of the washes. In each case, the rebinding of dissociated peptide-phage is prevented, and with increasing time, peptide-phage of higher and higher affinity are recovered. Additional modifications of the binding and washing procedures may be applied to find peptides which bind receptors under special conditions.

Although the phage screening method is highly specific, the procedure generally does not discriminate between peptides of modest affinity (micromolar dissociation constants) and those of high affinity (nanomolar dissociation constants or greater). The ability to select phage bearing peptides with relatively low affinity may be result of multivalent interaction between a phage/peptide particle and a receptor. For instance, when the receptor is an IgG antibody, each phage bearing peptides may bind to more than one antibody binding site, either by a single phage binding to both sites of single IgG molecule or by forming network of phage-IgG, which multivalent interaction produces a high avidity and tenacious adherence of the phage during washing.

To enrich for the highest affinity peptide ligands, a substantially monovalent interaction between phage and the receptor (typically immobilized on a solid-phase) may thus be appropriate. The screening (selection) with substantially monovalent interaction can be repeated as part of additional rounds of amplification and selection of bacteriophage. Thus, under these circumstances the receptor molecule is substantially monovalent, such as the Fab binding fragment of an antibody molecule.

A strategy employing a combination of conditions favoring multivalent or monovalent interactions can be used to advantage in producing new peptide ligands for receptor molecules. By conducting

the first rounds of screening under conditions to promote multivalent interactions, high stringency washing can be used to greatly reduce the background of non-specifically bound phage. This high avidity step may select a large pool or peptides with a wide range of affinities, including those with relatively low affinity. It may select for specific recognition kernels, such as the Tyr-Gly dipeptide described in the examples below. Subsequent screening under conditions favoring increasingly monovalent interactions and isolation of phage based on a slow dissociation rate may then allow the identification of the highest affinity peptides. Monovalent interactions may be achieved by employing low concentrations of receptor (for example, from about 1 to 100 pM).

It should be noted that, as an aspect of the present invention, determining a dissociation rate for a peptide of interest and the selected receptor molecule under substantially monovalent conditions allows one to extrapolate the binding affinity of the peptide for the receptor. This procedure avoids the necessity and inconvenience of separately determining binding affinities for a selected peptide, which could be especially burdensome if a large number of peptides have been selected.

Once a peptide sequence that imparts some affinity and specificity for the receptor molecule is known, the diversity around this "recognition kernel" may be embellished. For instance, variable peptide regions may be placed on one or both ends of the identified sequence. The known sequence may be identified from the literature, as in the case of Arg-Gly-Asp and the integrin family of receptors, for example, as described in Ruoslahti and Pierschbacher, Science 238:491-497 (1987), or may be derived from early rounds of panning in the context of the present invention.

Libraries of peptides on phage produced and screened according to the present invention are particularly useful for mapping antibody epitopes. The ability to sample a large number of potential epitopes as described herein has clear advantages over the methods based on chemical synthesis now in use and described in, among others, Geysen et al., J. Immunol. Meth. 102:259-274 (1987). In addition, these libraries are useful in providing new ligands for important binding molecules, such as hormone receptors, adhesion molecules, enzymes, and the like.

Accordingly, the following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

Reagents and Strains

BstXI restriction endonuclease, T4 DNA ligase, and T4 kinase were obtained from New England Biolabs. Streptavidin and biotinylated goat anti-mouse IgG were obtained from BRL. Sequenase 2.0 was obtained from U.S. Biochemical. Monoclonal antibody 3E7 used in initial studies was provided by A. Herz and is described in Meo et al., infra., incorporated herein by reference, and was also purchased from Gramsch Laboratories (Schwabhausen, West Germany). [^{125}I-tyr^{28}]b-endorphin (2000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Oligonucleotides were synthesized with an Applied BioSystems PCR-Mate and purified on OPC columns (ABI). Peptides were synthesized with an Applied BioSystems 431A (Foster City, CA) or Biosearch model 9600 (San Rafael, CA) synthesizer and purified to greater than 95% purity by reverse phage HPLC. Peptide content of the pure peptides was determined by amino acid analysis and the composition was verified by FAB-MS. Bacteriophage fd-tet and E. coli K91 were provided by G. Smith, Univ. of Missouri, Columbia, MO 65211, and are described in, among

others, Zacher et al., Gene 9:127-140 (1980), Smith et al., Science 228:1315-1317 (1985) and Parmley and Smith, Gene 73:305-318 (1988).

Construction of Vector fAFF1

A filamentous bacteriophage vector was constructed from the tetracycline resistance transducing vector fdTat, described in Zacher et al., supra. The vector, designated fAFF1, was designed to provide many choices in the size and location of the peptides expressed fused to the pIII bacteriophage coat protein. pIII is made as a preprotein with an 18 amino acid leader sequence that directs pIII to the inner membrane of the bacterial host cell before it becomes assembled into an intact phage particle (Goldsmith and Konigsberg, Biochem. 16:2686-2694 (1977) and Boeke and Model, Proc. Natl. Acad. Sci. USA 79:5200-5204 (1982) incorporated herein by reference). As explained further below, a peptide library was constructed by cloning an oligonucleotide of the structure shown in Fig. 1B to place the variable hexapeptide region at the N-terminus of the processed protein. These first six residues are followed by two glycines and then the normal sequence of pIII. The library consists of about 3 x 10^8 independent recombinants.

A cloning site, consisting of two non-complementary BstXI sites, was engineered into the 5'-region of gene III. As shown in Fig. 1A, two non-complementary BstXI sites flank the region encoding amino acids surrounding the signal peptidase site (the N-terminus of the mature pIII). fAFF1 also has a -1 frameshift mutation in pIII that results in non-infective phage. By removing the BstXI fragment and inserting an oligonucleotide of the appropriate structure, (a) portions of the removed sequence can be precisely reconstructed (the correct signal peptide site, for example,) (b) one or more additional amino acids may be

expressed at several locations, and (c) the correct translation frame is restored to produce active, infective pIII.

Construction of the cloning site at the 5'-region of gene III was accomplished by first removing a BstXI restriction site already present in the TN10 region of fdTet, RF DNA was digested with BstXI restriction endonuclease, and T4 DNA polymerase was added to remove the protruding 3' termini. Blunt-ended molecules were then ligated and transformed into MC1061 cells. RF DNA isolated from several tetracycline resistant transformants was digested again with BstXI; a clone that was not cleaved was selected for construction of the double BstXI site. Site-directed mutagenesis (Kunkel et al., Meth. Enzymol. 154:367-382 (1987), incorporated by reference herein) was carried out with the oligonucleotide 5'-TAT GAG GTT TTG CCA GAC AAC TGG AAC AGT TTC AGC GGA GTG CCA GTA GAA TGG AAC AAC TAA AGG. Insertion of the correct mutagenic sequence was confirmed by dideoxy sequencing of RF DNA isolated from several tetracycline-resistant transformants.

Construction of a Diverse Oligonucleotide Library

Oligonucleotides which were cloned have the general structure shown in Fig. 1B. The 5' and 3' ends have a fixed sequence, chosen to reconstruct the amino acid sequence in the vicinity of the signal peptidase site. The central portion contained the variable regions which comprise the oligonucleotide library members, and may also code for spacer residues on either or both sides of the variable sequence.

A collection of oligonucleotides encoding all possible hexapeptides was synthesized with the sequence 5'-C TCT CAC TCC $(NNK)_6$ GGC GGC ACT GTT GAA AGT TGT-3'. N was A, C, G, and T (nominally equimolar), and K was G and T (nominally equimolar). This sequence, designated ON-49, was ligated into the BstXI sites of fAFF1 after

annealing to two "half-site" oligonucleotides, ON-28 (5'-GGA GTG AGA GTA GA-3') and ON-29 (5'-CTT TCA ACA GT-3'), which are complementary to the 5'- and 3'- portions of ON-49, respectively. "Half-site" oligonucleotides anneal to the 5'- and 3'- ends of oligonucleotide ON-49 to form appropriate BstXI cohesive ends. This left the appropriate BstXI site exposed without the need to digest with BstXI, thus avoiding the cutting of any BstXI sites that might have appeared in the variable region. The vector fAFF1 (100 μg) was digested to completion with BstXI, heat inactivated at 65°C, and ethanol precipitated twice in the presence of 2 M ammonium acetate. Oligonucleotides were phosphorylated with T4 kinase, and annealed in 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, by mixing 1.5 μg ON-28, 1.2 μg ON-29, and 0.5 μg ON-49 with 20 μg BstXI-digested fAFF1 RF DNA, heating to 65°C for 5 minutes and allowing the mixture to cool slowly to room temperature. This represented an approximate molar ratio of 1:5:100:100 (fAFF1 vector: ON-49: ON-28: ON-29). The annealed structure is then ligated to BstXI-cut fAFF1 RF DNA to produce a double-stranded circular molecule with a small, single stranded gap. These molecules may be transformed into host cells. The annealed DNA was ligated in 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, by the addition of 20 units of T4 DNA ligase and incubated overnight at 15°C.

Alternatively, before transformation, the gap may be filled-in under conditions disfavoring secondary structure in the variable region. In some experiments the gapped circular structure created by this ligation was filled in with T4 DNA polymerase in the presence of ligase and dNTPs (400 μM each) to produce a covalently closed, double-stranded molecule (Kunkel et al., supra). The ligated DNA was ethanol precipitated in the presence of 0.3 M sodium acetate, resuspended in water, and transformed by electroporation into MC1061. Five electro-transformations, each containing 80 μl of cells

and 4 μg of DNA (50 μg/ml), were performed by pulsing at 12.5 kV/cm for 5 msec as described in Dower et al., Nucleic Acids Res. 16:6127-6145 (1988), incorporated by reference herein. After one hour of non-selective outgrowth at 37°C in 2 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose; see Hanahan, J. Mol. Biol. 166:557-580 (1983)), the transformations were pooled, an aliquot was removed, and several dilutions were plated on LB agar plates containing tetracycline (20 μg/ml) to assess the transformation efficiency. The remainder was used to inoculate one liter of L-broth containing tetracycline (20 μg/ml) and was grown through approximately 10 doublings at 37°C to amplify the library.

Isolation of Phage

Phage from liquid cultures were obtained by clearing the supernatant twice by centrifugation (8000 RPM for 10 min in JA10 rotor, at 4°), and precipitating phage particles with polyethylene glycol (final concentration 3.3% polyethylene glycol-8000, 0.4 M NaCl), and centrifuged as described above. Phage pellets were resuspended in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and stored at 4°C. In some cases, phage were isolated from plate stocks by scraping from the agar surface, resuspending in L-broth, and purifying as described above.

Affinity Purification

Approximately 10^3 to 10^4 library equivalents of phage were reacted overnight with 1 μg of purified antibody in 1 ml TBS at 4°C. (Under these conditions, phage and antibody are about equimolar; therefore, antibody is in great excess over the phage ligand peptides.) Phage expressing peptides with affinity for mAb3E7 were isolated by a modification of the procedure

of Parmley and Smith, supra. A 60 x 15 mm polystyrene petri plate was coated with 1 ml of streptavidin solution (1 mg/ml streptavidin in 0.1 M $NaHCO_3$, pH 8.6, 0.02% NaN_3) and incubated overnight at 4°C. The streptavidin solution was removed the following day. The plate was filled with 10 ml blocking solution (30 mg/ml BSA, 3 µg/ml streptavidin in 0.1 M $NaHCO_3$, pH 9.2, 0.02% NaN_3) and incubated for two hours at room temperature. Biotinylated goat anti-mouse IgG (2 µg) was added to the antibody-reacted phage library and incubated for two hours at 4°C. Immediately before panning, the blocking solution was removed from the streptavidin-coated plate, and the plate was washed 3 times with TBS/0.05% Tween 20. The antibody-reacted phage library was then added to the plate and incubated for 30 min. at room temperature. The phage solution was removed and the plate was washed ten times with 10 ml TBS/0.05% Tween 20 over a period of 60 min. at room temperature. Adherent phage were removed by adding 800 µl of elution buffer (1 mg/ml BSA in 0.1 N HCl adjusted to pH 2.2 with glycine) to the petri plate and incubating for 10 min. to dissociate the immune complexes. The eluate was removed, neutralized by addition of 45 µl of 2 M Tris base, and used to infect log phase E. coli K91 cells.

The infected cells were then plated on LB agar plates containing tetracycline (20 µg/ml), and grown overnight at 37°C. Phage were isolated from these plates as described above and the affinity purification process was repeated for two more rounds. After each round of panning and amplification, DNA of phage from several thousand colonies was pooled and sequenced to estimate the diversity in the cloning site. In the first two positions of each codon, bands of about the same intensity appeared in each lane, indicating the expected distribution of bases in these positions. In the third position of each codon the G band was somewhat more intense than the T band.

After the final round of panning and amplification, a portion of the eluate was used to infect cells that were plated at low density on LB tetracycline plates. To analyze the diversity of peptide sequences in the library in a more direct way, we picked 52 individual colonies producing infectious phage, and sequenced the DNA of their variable regions. Individual colonies were picked and transferred to culture tubes containing 2 ml LB tetracycline and grown to saturation. Phage DNA was isolated and then sequenced by a method designed for the Beckman Biomek Workstation employing 96-well microtiter plates (Mardis and Roe, Biotechniques 7:840-850 (1989), incorporated by reference herein). Single stranded DNA was sequenced using Sequenase 2.0 and an oligonucleotide sequencing primer (5'-CGA TCT AAA GTT TTG TCG TCT-3') which is complementary to the sequence located 40 nucleotides to the 3' side of the second BstXI site in fAFF1.

The distribution of bases at each position within each codon is given in Table 1. The first two positions of each codon have close to the expected equimolar distribution of the four bases. The third position is significantly biased, containing about 50% more G than T in this sample. This bias is most likely introduced during the chemical synthesis of the oligonucleotide mixture, but may also reflect biological biases imposed on the expressed peptides.

Table 1: Nucleotide Distribution in the Diversity Region of Infectious Phage Randomly Selected from the Library.

	Frequency of each base by position in codon (%)		
	N	N	K
G	31	27	59
A	22	22	<1
T	25	26	39
C	22	24	1

In Fig. 2, the amino acid sequences are listed for the peptides encoded by the oligonucleotide inserts of a sample of randomly selected, infectious phage. The amino acid content of the expressed peptides from the 52 randomly selected infectious phage appears in Table 2.

Table 2: Amino Acid Content in the Variable Peptide of 52 Randomly-Selected Infectious Phage

Amino Acid	Nominal Frequency	Nominal Occurrence	Observed Occurrence	Obs/Nom
A	0.065	19	27	1.42
C	0.032	9	8	0.89
D	0.032	9	10	1.11
E	0.032	9	9	1.00
F	0.032	9	12	1.33
G	0.065	19	33	1.74
H	0.032	9	7	0.78
I	0.032	9	6	0.67
K	0.032	9	16	1.78
L	0.097	28	35	1.25
M	0.032	9	10	1.11
N	0.032	9	7	0.78
P	0.065	19	9	0.47
Q	0.032	9	15	1.67
R	0.097	28	29	1.04
S	0.097	28	30	1.07
T	0.065	19	14	0.74
V	0.065	19	18	0.95
W	0.032	9	11	1.22
Y	0.032	9	6	0.67

As shown in Table 2, the ratio of the observed occurrence of each amino acid to that expected on the basis of codon frequency ranges from about 0.5 to 2, consistent with a random distribution of sequences.

Constructing a library of peptides displayed on the N-terminus of processed pIII necessarily alters amino acids in the vicinity of the signal peptidase cleavage site. Certain changes in the corresponding region of the major coat protein, pVIII, have been shown to reduce processing efficiency, slowing or preventing the incorporation of pVIII to virions. If pIII were similarly affected, the diversity of peptides contained in the library would be reduced. The finding that most amino acids appear at each position of the variable peptides of randomly selected phage indicates that processing defects do not impose severe constraints on the diversity of the library.

Isolation and sequencing of phage having high avidity for anti-b-endorphin antibody.

Monoclonal antibody 3E7 binds to B-endorphin and, like the δ-opioid receptor, recognizes the N-terminal portion of the protein (Tyr-Gly-Gly-Phe), which is present on most natural opioid peptides. The antibody also binds tightly to leu- and met-enkephalin (YGGFL, YGGFM), and a variety of related opioid peptides (Meo et al., Proc. Natl. Acad. Sci. USA 80:4084-4088 (1983), Herz et al., Life Sciences 31:1721-1724 (1982), and Gramsch et al., J. Neurochem. 40:1220-1226 (1983). The N-terminal hexapeptide library was screened against 3E7 by carrying out three rounds of panning, elution, and amplification. The recoveries of phage from this process are shown in Table 3. In each round the proportion of phage adsorbed to the antibody increased by about 100-fold, and in the last round, over 30% of the input phage were recovered. These results indicated that phage were preferentially enriched in each panning step.

Table 3: Recovery of Phage from Panning on mAb3E7

Rounds of Panning	Input of Phage	Eluted Phage	Recovery Input/Eluted
1	4.0×10^{11}	1.9×10^{7}	4.8×10^{-5}
2	2.0×10^{11}	5.0×10^{8}	2.5×10^{-3}
3	1.8×10^{10}	5.6×10^{9}	3.1×10^{-1}

After each round of panning, DNA representing several thousand eluted phage was pooled and sequenced. The area of the sequencing gel corresponding to the insertion site in gene III is shown in Fig. 3. The codon TCC specifying the serine that precedes the variable region is indicated by an arrow. After the first round of panning, the codon following this serine was clearly enriched in TAT (the single codon for tyrosine). After the second round, virtually all first codons in the pooled DNA appeared to be TAT. The second codons are strongly GGK (the two codons for glycine). After three rounds of panning, it appeared that phage containing relatively few kinds of amino acids in the first four positions had been selected, whereas the fifth and sixth positions appeared to be as diverse as those in the starting phage population.

The DNA samples from 51 individual phage recovered from the third panning were sequenced. The deduced amino acid sequences of the N-terminal hexapeptides are shown in Fig. 4 and the amino acid distributions of these peptides are summarized in Table 4. Each of the 51 panned phages analyzed had an N-terminal tyrosine, and nearly all (94%) had a glycine in the second position. The third position in our sample is occupied by many amino acids, some of which are present

more often than would be expected by chance. The fourth position is occupied primarily by the large aromatic residues Trp and Phe (together 50%), and the bulky hydrophobic residues Leu and Ile (an additional 45%). The fifth and sixth positions contain essentially random distributions of amino acids, with only alanine appearing at slightly greater than chance in position five.

Table 4: Distribution of Amino Acids in the Diversity Peptide of 51 Phage Selected by Panning With Anti-endorphin Antibody

Residue Position	Amino Acid	Nominal Frequency	Observed Frequency	Enrichment[a] Ratio
1	Y	.031	1.00	33
2	G	.062	0.94	16
	A,S			<1
3	G	.062	0.31	5
	W	.031	0.10	3
	S	.093	0.21	2
	A	.062	0.12	2
	N	.031	0.06	2
	D,E,F,K, L,M,P,T,V			<1
4	W	.031	0.31	10
	F	.031	0.19	6
	L	.093	0.35	4
	I	.031	0.10	3
	A,G,M			<1

a. Observed frequency divided by nominal frequency.

EXAMPLE II

Binding Affinities of Peptides for Receptor Monoclonal Antibody 3E7

The affinity of peptides for the 3E7 antibody has been previously determined for those peptides related to naturally-occurring opioid peptides. Meo et al., supra. As none of the peptides identified by the procedure described herein had been previously described, six of these peptides were chemically synthesized and their binding affinities estimated.

The peptides were synthesized according to well known protocols, as described in, for example, Merrifield, Science 232:341-347 (1986), incorporated by reference herein. A solution radioimmunoassay was used to estimate the binding affinities of peptides for mAb 3E7. Solution radioimmunoassay using [^{125}I]b-endorphin (20,000 cpm) and purified 3E7 antibody (0.25 µg/ml) was conducted as described by Meo et al., supra, with the exception that the final volume was 150 µl. Antibody-bound and free [^{125}I]b-endorphin were separated by addition of activated charcoal followed by centrifugation, as described in Ghazarassian et al., Life Sciences 27:75-86 (1980). Antibody-bound [^{125}I]b-endorphin in the supernatant was measured in a gamma counter. For each peptide, inhibition of [^{125}I]b-endorphin was determined at six different concentrations at 1/3 log unit intervals and the 50% inhibitory concentration (IC50) was determined by fitting the data to a two-parameter logistic equation using the ALLFIT program, as described in DeLean et al., Am. J. Physiol. 235:E97-E102 (1978).

The previously reported high degree of specificity of the 3E7 antibody for the intact N-terminal epitope Tyr-Gly-Gly-Phe which is common to naturally occurring opioid peptides. Meo et al., supra, was verified. Removal of Tyr or deletion of any of the amino acids of the sequence Tyr-Gly-Gly-Phe-Leu had deleterious effect on binding affinity (Table 5).

Shown in Table 5 are the IC50 for the six peptides which were identified by the phage panning method and chemically synthesized. Under the conditions of the radioimmunoassay (30 pM [^{125}I]b-endorphin; 20% tracer bound; 18 hr. incubation), the IC50 should be very close to the dissociation constant (Kd) for the peptide. The peptides are all relatively low affinity compared to YGGFL, with IC50's ranging from 0.35 to 8.3 μM.

Table 5: Relative affinities of peptides for 3E7 antibody determinod by solution radioimmunoassay.[a]

Peptide	N	IC50 (μM)		Affinity Relative to YGGFL
YGGFL	(6)	0.0071	(0.0054,0.0093)	1
YGGF	(3)	0.19	(0.093,0.38)	0.037
YGGL	(3)	3.8	(2.1,6.6)	0.0018
YGFL	(3)	28	(17,47)	0.00025
YGG	(2)	>1000		<0.0000071
GGFL	(2)	>1000		<0.0000071
GGF	(2)	>1000		<0.0000071
GFL	(2)	>1000		<0.0000071
YGFWGM	(3)	0.35	(0.19,0.63)	0.020
YGPFWS	(3)	1.9	(1.3,2.8)	0.0037
YGGFPD	(3)	2.3	(1.4,3.7)	0.0031
YGGWAG	(3)	7.8	(6.0,10)	0.00091
YGNWTY	(3)	7.8	(4.0,15)	0.00091
YAGFAQ	(3)	8.3	(3.8,18)	0.00086

[a] = Data are geometric means and 95% confidence intervals (calculated from S.E.M. of log IC50) from the number (N) of independent determinations indicated.

The data indicate that although the phage panning method is highly specific in that no unrelated peptides were selected, the procedure apparently does not discriminate between those of moderate (μM Kd) and high (nM Kd) affinity. The six peptides chosen from among the 51 clones that were sequenced were only a small subset of those which were selected by three rounds of panning. Based on their structural diversity, the phage library

should contain thousands of different peptides with dissociation constants that are μM or lower.

The panning procedure we have utilized employs extensive washing to remove non-specifically bound phage. Binding experiments with mAb 3E7 and [^{3}H]YGGFL indicate a rapid dissociation rate, approximately $t_{\frac{1}{2}}$=45 seconds at room temperature. Therefore, the ability to select phage bearing peptides with relatively low affinities may be the result of multivalent interaction between phage and antibody, as each phage typically has up to 4 or 5 copies of the pIII protein and each protein may carry a foreign peptide from the phage library.

EXAMPLE III

Selective Enrichment and Characterization of High Affinity Ligands from Collections of Random Peptides on Phage

Phage bearing peptides YGGFL and YAGFAQ served as models to determine the effect of IgG and Fab concentration on the binding and recovery of phage bearing high (nM Kd) and low (μM Kd) affinity peptides.

To determine the effect of polyvalency, a phage sandwich ELISA was developed which used polyclonal anti-phage antibodies to detect bound phage. Purified monoclonal antibody 3E7 was used as intact IgG and as Fab fragments (produced using a commercially available kit (Pierce), and biotinylated Fab fragments (Gramsch Laboratories). No IgG was detected in Fab preparations when they were run on SDS-PAGE gels and stained with Coomassie blue. Fab was iodinated by reacting 5μg of Fab in 20 μl of 0.1M borate buffer (pH 8.5) with 250 μCi of [^{125}I]Bolton-Hunter reagent (Amersham) for 3 hours and then purified by gel filtration on Sephadex G25. After

purification, the specific activity of the [^{125}]FAB was approximately 15 μCi/μg.

Antisera were raised against phage particles lacking pIII fAFF1, which, as described above, contains a frameshift in the 5' end of gene III and is produced as non-infective polyphage. Cells from a two liter culture of E. coli K91 were removed by centrifugation and media was mixed with 400 ml of 20% PEG in 0.5M NaCl. After incubation for 1 hr at 4 C. precipitated phage were isolated by centrifugation at 8500 rpm. The pellet was resuspended in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then ultracentrifuged in a SW50 rotor at a 42,000 rpm for 3 hrs. The resulting pellet was resuspended in water and the concentration of phage was estimated according to the method of Day, J. Mol. Biol. 39:265 (1969).

Three rabbits were injected intramuscularly with 0.5 mg of phage in Freunds complete adjuvant and then boosted with 0.25 mg of phage in incomplete adjuvant at 3 week intervals. The titer of the sera was measured with an ELISA using phage immobilized in Immulon 2 microtiter wells as described above. All rabbits produced high titer sera after the second boost. Sera collected after the third boost from one of the rabbits was used for the assays.

Antibodies reacting with phage were affinity purified as follows. Phage expressing native pIII (Fd-tet) from a two liter culture were isolated (described above) and added to 20 ml of sera that was diluted 4-fold with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). After incubation for 2 hr at room temperature, phage/antibody complex was isolated by centrifugation for 1 hr at 120,000 x g. The pellet was washed with 10 ml of PBS and centrifuged again. The final pellet was resuspended in 10 ml of 100 mM sodium acetate buffer pH 2.5 and incubated for 10 min. at room temp. The sample was subjected to same centrifugation and the resulting supernatant was neutralized with NaOH. IgG was then

isolated using Protein-A agarose (Pierce) according to the manufacturer's instructions. The IgG was conjugated to alkaline phosphatase using a commercially available kit (Pierce).

The phage sandwich ELISAs were performed as follows. Microtiter wells were incubated with 100 μg/ml of streptavidin for 1 hr at 37 °C, then blocked with 200 μl of PBS /0.1% bovine serum albumin (BSA) for 1 hr. Biotinylated IgG (0.5 μg/ml) or biotinylated Fab (5 μg/ml) (100 ml in PBS/0.1% BSA) was added to the wells and incubated for an additional 1 hr at room temperature. Preliminary studies in which immobilized IgG or Fab was detected with goat anti-mouse IgG conjugated to alkaline phosphatase indicated that these conditions maximally saturated the well with IgG or Fab. The difference in concentration for biotinylated IgG and Fab required for saturation of the streptavidin probably reflected differences in the fraction of the protein that was biotinylated.

After washing the wells with PBS, 50 μl of PBS/0.1% BSA/0.05% Tween 20 or the same buffer containing 200 μM YGGFL free peptide was added to the well and incubated for 20 min at room temperature. Phage (10^{10} infectious particles in 50 μl PBS/0.1% BSA/0.05 Tween 20) bearing the peptides YGGFL or YAGFAQ were added and incubated for 18 hr at 4 C. After washing with TBS/0.05% Tween 20, alkaline phosphatase anti-phage antibody (100 μl of 1:100 dilution) was added and incubated for 1 hr. at room temperature. After washing with TBS/0.05% Tween 20, 100 μl of alkaline phosphatase substrate (SIGMA) in diethanolamine buffer (pH 9.5) was added and the absorbance at 405 nm was measured 10 min. later.

Shown in Fig. 5 are the results of this assay when either biotinylated IgG or Fab was immobilized at maximal density on streptavidin coated wells. Specific binding was detected for both YGGFL- and YAGFAQ-phage; the data indicate that the amount of binding did not

differ when IgG and Fab were used. In combination with the data on monovalent dissociation rates of these peptides (see below), this suggests that antibody binding sites for both IgG and Fab are in sufficient proximity to one another to allow simultaneous binding of more than one of the peptides expressed by each phage particle.

The phage sandwich assay can also be used to determine the specificity and competitive nature of the interaction of peptide-bearing phage with immobilized antibody. In practice, an important aspect of the use of peptide on phage libraries is the characterization of individual phage isolates after sequential rounds of the affinity purification. Isolated phage may bind to other components found on an immobilizing surface, or may bind to the protein target at sites other than the active site. Using the phage sandwich assay, the binding of YGGFL- and YAGFAQ-phage was shown to be specific for the antibody and the interaction of the phage with antibody could be blocked by free YGGFL peptide.

Shown in Fig. 6 are the results of tests on the effect of Fab concentration and wash time on the recoveries of YGGFL- and YAGFAQ-phage. Microtiter wells were coated with streptavidin as described above. 10^{11} infectious phage particles bearing the peptides YGGFL or YAGFAQ were incubated overnight at 4°C with either 50 µl of 5nM or 50 pM biotinylated Fab. Aliquots were then added to different microtiter wells and incubated for 1 hr. at room temperature. All the wells were washed quickly with TBS/0.05% Tween 20, with the last 200 µl wash being left in the well. At various times thereafter, wells washed quickly with TBS/0.05% Tween and the phage remaining bound were eluted with 0.1 M HCl (pH adjusted to 2.2 with glycine) and quantitated by titering as described above.

The results indicate that low Fab concentration (50pM) and dissociation times greater than 30 minutes allowed the selective recovery of phage bearing the

higher affinity peptide YGGFL. The use of a high concentration of Fab (5 nM) did not allow the discrimination of phage bearing high and low affinity peptides.

Phage enrichment using a low concentration of biotinylated 3E7 Fab.

A pool of phage previously isolated by three rounds of panning and amplification using 5 nM 3E7 IgG served as starting material for additional affinity purification and amplification using a modification of the previous protocol. Phage (10^{11} infectious particles in 1 ml of TBS) were incubated overnight at 4°C with 2 ng of biotinylated Fab (50 pM final concentration). The mixture was then exposed to streptavidin-coated plates and bound phage were isolated as described above. Individual phage clones were then isolated and DNA was sequenced as described in Example I above.

Shown in Table 6 are the sequences of peptide inserts of phage that were isolated by 2 rounds of affinity isolation and amplification using 50 pM of biotinylated Fab. A notable difference between the sequences shown in Table 5 and those identified in Example II using three rounds of affinity purification with 50 nM IgG, is the frequency of Phe in the fourth position: 13/19 vs. 10/51 ($p<0.05$ Fisher Exact Test). Thus, the sequences more highly resemble the known high affinity peptides YGGFL and YGGFM. The sequence YGGFLT was isolated by this procedure and, of the 20 clones that were selected from the final pool of phage, there was

only one repeat (the nucleotide sequence was also identical).

TABLE 6

	Dissociation t1/2 (minutes)	Equilibrium IC50 (nM)
Control Peptides		
YGGFL *	18.2	6.6 ± 3.5
YGFWGM*	0.25	150
YAGFAQ*	**	8300
Peptides isolated with 50pM Fab		
YGAFQG	18.9	27 ± 2.0
YGGFLT	17.9	
YGYWSL	15.6	
YGAFMQ	13.7	13 ± 4.9
YGAFFQ	13.4	
YGAFFK	9.1	59 ± 22
YGFWSN	7.4	
YGAFGG	5.0	
YGGFGF	4.7	65 ± 18
YGVFSR	2.8	
YGGLSM	0.96	
YGTFLN	0.75	470 ± 140
YGGLVR	0.50	
YGSFSL	0.43	
YGAWYT	**	1600 ± 300
YGRFFH	**	
YGGLRH	**	
YGSFMA	**	
YGGFSP	**	

** indicates that initial binding was not detected

Determination of the dissociation of [^{125}I]Fab from fusion phage clones.

An assay employing the anti-phage antisera was developed to determine the rate of dissociation of [^{125}I]Fab from individual phage isolates. Individual

fusion phase isolates were amplified in a 5 ml liquid culture of E. coli K91 cells and phage particles were isolated and quantitated as described above. In 1 ml microtiter minitubes, 25 µl of TBS containing approximately 2 x 10^9 infectious phage particles were incubated with 25 ml of TBS/0.1% BSA containing 40,000 cpm of [^{125}I]Fab for 10 minutes. Anti-phage antisera (25 µl of 1:1000 dilution in PBS/0.1%BSA) and staph A particles coated with goat anti-mouse IgG (25 µl of Tachisorb diluted eight-fold in TBS/0.1% BSA) were then added. Dissociation was initiated after 2 hours further incubation at 4°C. To prevent binding of unbound [^{125}I]Fab, 25 µl of a 400 µM solution of YGGFL in PBS/0.1% BSA was added to all tubes and the amount of phage-bound [^{125}I]Fab was determined by automated filtration on glass fiber filters previously treated with 1% BSA. Filter bound radioactivity was determined by gamma counting. Binding was determined in triplicate prior to and 0.5, 1, 2, 4, 8, 16, 32 minutes after the addition of YGGFL peptide. The time corresponding to a 50% reduction of initially bound [^{125}I]Fab was determined by linear regression of a semi-logarithmic plot of amount bound vs. time. This assay was calibrated with library phage clones bearing peptides of known affinity (YGGFL, 7nM and YGFWGM, 350 nM), as shown in Fig. 7.

Shown in Table 6 are the t1/2 values for the phage clones that were picked from the pool of phage isolated using 5 pM Fab. Several of the clones had t1/2 values similar to the control phage YGGFL. Specific binding of [^{125}I]Fab was not detected for 5 of the 20 clones that were examined.

For a series of related ligands, the rank order of dissociation rates should correlate with the rank order of equilibrium binding constants (Kds). This correlation was confirmed and a quantitative relationship established between dissociation rates and the Kd of the corresponding free peptide. In addition, the affinity

requirements for selection using low Fab concentrations were established.

Several peptides corresponding to those phage clones with differing dissociation rates were chemically synthesized and their potencies were determined in a solution competition assay. The t1/2 values correlated with the IC50 of the corresponding free peptide. Under the conditions of the competition assay (low concentration of tracer, <20% bound tracer, 18 hr. incubation), the IC50 should approximate the Kd. For phage bearing peptides with Kds greater than 500 nM, specific binding was not detected under these monovalent assay conditions.

EXAMPLE IV

Conotoxin Peptide Libraries Having Conserved Disulfide Frameworks.

A conotoxin peptide library is prepared as generally described above, by synthesizing oligonucleotides containing degenerate codons of the NNK (or NNS) motif. Here N is equimolar A,C,G, or T, and K is equimolar G or T (S=G or C). This motif codes for all 20 amino acids at each locus in the hypervariable regions. (Alternatively, the degenerate portion can be assembled by the condensation of 20 activated trinucleotides, one for each amino acid.) The six cysteine codons are preserved to produce the characteristic conotoxin frameworks.

To sample additional diversity in the peptide libraries, the number of residues between the Cys's is varied. This is accomplished as follows:

(1) Five separate oligonucleotide synthesis columns are prepared with the first nucleotide immobilized on resin. (2) The common regions of the 3' end of the oligonucleotides is synthesized (all columns

go through the same cycles to produce the cloning site, etc., on this end). Synthesis on all columns is carried out through the first Cys (or CysCys) of the cono-framework. (3) On column 1, two degenerate codons are synthesized; on column 2, three degenerate codons are synthesized, on so on. Each column now has oligonucleotides with either 2,3,4,5, or 6 degenerate codons in the first hypervariable region. (4) One Cys codon is now added to all columns (this is the second Cys of the omega class or the third Cys of the mu class). (5) The resins from all five columns are removed, mixed well, and reallocated among the five columns. Each column now contains oligonucleotides with all five lengths of first hypervariable region. (6) Each column is again put through either 2,3,4,5, or 6 cycles of degenerate codon synthesis as before; and the next Cys codon (or CysCys for omega) is added. (7) The resins are again removed, mixed, and redistributed to the five columns, and the process is repeated through three (for mu) of four (for omega) hypervariable regions. (8) The common sequence on the 5' end of all the oligonucleotides is synthesized, and the oligonucleotides are removed from the resins and purified as usual.

Folding of the peptides to achieve biological activity may be directed by a 40 amino acid conserved "leader peptide" at the N-terminus of the pretoxin molecule. Synthesized as part of a recombinant fusion protein, this leader may enhance the folding of many of the members of the library into the "correct" conotoxin-like framework. Alternatively, allowing the cysteine framework to form in a random manner produces a variety of structures, only some of which mimic the conotoxin framework. This collection provides additional multi-loop structures that add to the diversity of the peptide library.

To minimize the possibility that one conformation would predominate, a gentle reduction of the

phage in vitro is employed, followed by mild oxidation to form most of the conformations. Mild reduction/oxidation can be accomplished by treatment with 0.2 to 5 mM DTT followed by extensive dialysis to non-reducing conditions. A regenerable, immobilized lipoic acid column to rapidly pass the peptide-bearing particles over can also be used.

The possibility of promiscuous binding of Cys residues in the peptide binding to other proteins can also be minimized by mild reduction and oxidation, or can be avoided by re-engineering the fusion protein by site-directed mutagenesis to remove the Cys residues.

Peptides with the conotoxin framework can be expressed in several types of libraries as described herein. For example, the peptides can be 1) expressed in an N-terminal library in phage fAFF1; 2) expressed internally, fused to pIII at or near the N-terminus, displacing the degenerate peptides 2 to 10 or more residues from the cleavage point to circumvent processing problems; 3) expressed in a carboxy terminal exposed library (as many of the conotoxins are C-terminally amidated, residues with amino side chains can be added to the C-terminal end of the peptides, or the peptide library, can be amidated in vitro); and 4) the putative 40 residue "folding peptide" can be installed upstream of degenerate peptides displayed in the C-terminally exposed configuration.

This general format for using the secondary framework structure of conotoxins can also be applied to other peptide families with biological activities as a basis for designing and constructing peptide expression/screening libraries in accordance with the present invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will

be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for identifying peptides of interest which bind to a preselected receptor, comprising:

transforming host cells with a bacteriophage expression vector which comprises an oligonucleotide library of at least about 10^6 members which encode peptides, wherein a library member is joined in reading frame to the 5' region of a nucleotide sequence encoding an outer structural protein of the bacteriophage;

cultivating the transformed host cells under conditions suitable for expression and assembly of bacteriophage;

contacting bacteriophage that express the peptides to the preselected receptor under conditions conducive to specific peptide-receptor binding; and

selecting bacteriophage which bind to the receptor and therefrom identifying the peptides of interest.

2. The method of claim 1, further comprising the step of determining the nucleotide sequence encoding the peptide of interest in the selected bacteriophage.

3. The method of claim 1, wherein the selected bacteriophage are propagated and the contacting and selecting steps are repeated to enrich for bacteriophage which express the peptides of interest.

4. The method of claim 3, wherein the valency of the specific peptide-receptor binding interaction is reduced in subsequent repetitions of the contacting, selecting and propagating steps to enrich for peptides of higher binding affinity.

5. The method of claim 1, wherein the bacteriophage expressing peptides and receptor are present at concentrations that produce a substantially monovalent

binding interaction between the receptor and bacteriophage which express peptides of interest.

6. The method of claim 5, wherein the contacting step under substantially monovalent binding conditions is repeated at least once between additional rounds of propagation of the selected bacteriophage.

7. The method of claim 6, wherein the receptor is monovalent.

8. The method of claim 7, wherein the monovalent receptor is a Fab fragment of an antibody.

9. The method of claim 1, further comprising determining a dissociation rate for the peptide of interest and the receptor under substantially monovalent peptide-receptor binding conditions and therefrom determining the binding affinity of the peptide of interest for the receptor.

10. The method of claim 1, where the receptor is bound to a solid phase and the selected bacteriophage are separated from the culture.

11. The method of claim 10, wherein said receptor is an antibody or binding fragment thereof.

12. The method according to claim 1, wherein the outer protein is a bacteriophage coat protein.

13. The method of claim 1, wherein the bacteriophage encoded by the expression vector is a filamentous phage.

14. The method of claim 13, wherein the filamentous bacteriophage is f1, fd, or M13.

15. The method of claim 14, wherein the bacteriophage is fd or a derivative thereof.

16. The method of claim 15, wherein the outer bacteriophage protein is a coat protein.

17. The method of claim 16, wherein the coat protein of the fd bacteriophage is pIII.

18. The method of claim 1, wherein the oligonucleotide library comprises a series of codons encoding a random collection of amino acids.

19. The method of claim 18, wherein the codons encoding the collection of amino acids are represented by $(NNK)_x$ or $(NNS)_x$, where N is A, C, G or T, K is G or T, S is G or C, and x is from 5 to 8.

20. The method of claim 19, wherein the series of codons encoding the random collection of amino acids of the oligonucleotide library member encodes a hexapeptide.

21. The method of claim 19, wherein x is 8 and the recombinant bacteriophage screened in the selecting step represents up to about 10% of the possible octapeptides.

22. The method of claim 18, wherein the oligonucleotide library member further encodes at least one spacer residue.

23. The method of claim 22, wherein a spacer residue comprises Gly.

24. The method of claim 23, wherein the spacer comprises Gly-Gly.

25. The method of claim 18, wherein the oligonucleotide library is flanked by nucleotide sequences that encode conserved residues that comprise structural frameworks for peptides of interest.

26. The method of claim 25, wherein the flanking sequences encode Cys residues.

27. The method of claim 26, wherein the Cys residues flank the N- and C- terminals of the peptide of interest.

28. The method of claim 26, wherein at least one conserved Cys residue is encoded within the library variable region.

29. The method of claim 25, wherein the structural framework comprises that of a conotoxin-like peptide and the conserved residues are Cys.

30. The method of claim 29, wherein the sequence of the structural framework comprises Cys-Cys-Y-Cys-Y-Cys-Cys or Cys-Y-Cys-Y-Cys-Cys-Y-Cys-Y-Cys, wherein Y is $(NNK)_x$ or $(NNS)_x$, where N is A, C, G or T, K is G or T, S is G or C, and x is from 2 to 6.

31. The method of claim 18, wherein the variable codon region is prepared from a condensation of activated trinucleotides.

32. The method of claim 18, wherein fragments of 20 to 100 basepairs of a gene which encodes a known ligand for the preselected receptor are cloned into the oligonucleotide library.

33. The method of claim 1, wherein the host cells are transformed by electroporation.

34. The method of claim 1, wherein the oligonucleotide library comprises at least about 10^8 members.

35. The method of claim 1, wherein the oligonucleotide library members are inserted in the bacteriophage expression vector so that the N-terminus of the processed bacteriophage outer protein is the first residue of the peptide.

36. The method of claim 1, wherein the bacteriophage protein is a preprotein which is processed by the host cell to leave the peptide encoded by an oligonucleotide library member exposed at the N-terminus of the mature outer structural protein.

37. The method according to claim 36, wherein the peptide comprises spacer amino acid residues are encoded by the oligonucleotide library members between the N-terminus of the mature outer protein and the C-terminus of the peptide.

38. A method for identifying peptides of interest which bind to a preselected receptor, comprising:

transforming host cells with a bacteriophage expression vector which comprises an oligonucleotide library which encodes peptides, wherein a library member is joined in reading frame with a nucleotide sequence to encode a fusion protein, wherein the library member represents the 5' member of the fusion protein and the 3' member comprises at least a portion of an outer structural protein of the bacteriophage;

cultivating the transformed cell under conditions suitable for expression and assembly of bacteriophage;

contacting bacteriophage that express the peptides to the preselected receptor under conditions conducive to specific peptide-receptor binding; and

selecting bacteriophage which bind to the receptor and therefrom identifying the peptides of interest.

39. A composition comprising a peptide produced according to the method of claim 1, 13, 19, 31 or 38.

40. The composition of claim 39, wherein the peptide binds an antibody.

41. An oligonucleotide library produced according to claims 1, 13, 19, 31 or 38.

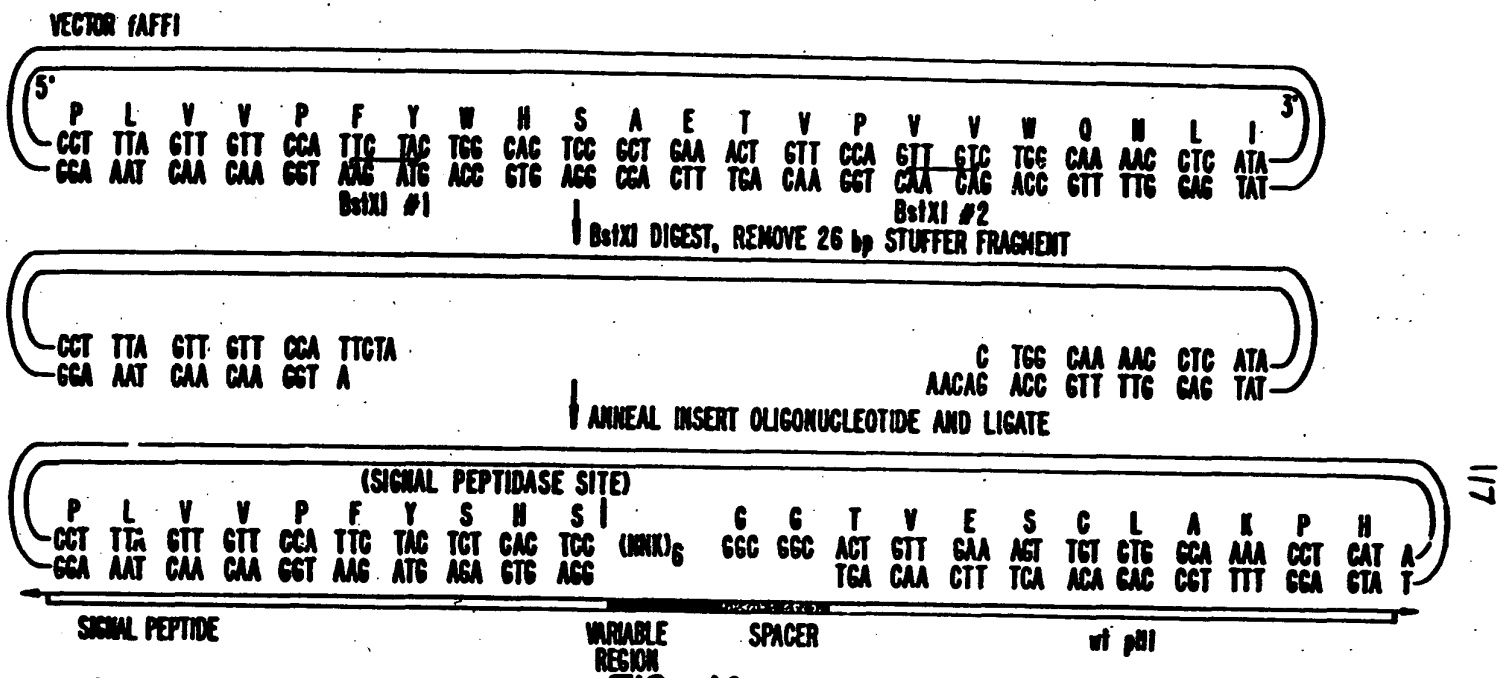
42. A host cell transformed with a bacteriophage expression vector which comprises an oligonucleotide library member, joined in reading frame to the 5' region of a nucleotide sequence encoding an outer structural protein of the bacteriophage, wherein the library member encodes a peptide of at least about five to twenty-five amino acids.

43. The host cell of claim 42, wherein the oligonucleotide library member comprises a series of codons encoding a random collection of from five to eight amino acids.

44. The host cell of claim 43, wherein the oligonucleotide library member further comprises a sequence encoding at least about one to five spacer amino acids which are expressed adjacent to the random collection of amino acids.

45. A collection of filamentous bacteriophage having a peptide on the N-terminus of a coat protein, wherein the peptide is coded for by a oligonucleotide

library member from a randomly generated mixture of oligonucleotides.


VECTOR fAFF1
5'
3'
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CCT TTA GTT GTT CCA TTC TAC TGG CAC TCC GCT GAA ACT GTT CCA GTT GTC TGG CAA AAC CTC ATA
GGA AAT CAA CAA GGT AAG ATG ACC GTG AGG CGA CTT TGA CAA GGT CAA CAG ACC GTT TTG GAG TAT
BstXI #1
BstXI #2
BstXI DIGEST, REMOVE 26 bp STUFFER FRAGMENT
CCT TTA GTT GTT CCA TTCTA
GGA AAT CAA CAA GGT A
C TGG CAA AAC CTC ATA
AACAG ACC GTT TTG GAG TAT
ANNEAL INSERT OLIGONUCLEOTIDE AND LIGATE
(SIGNAL PEPTIDASE SITE)
P L V V P F Y S H S (NNK)6 G G T V E S C L A K P H
CCT TTA GTT GTT CCA TTC TAC TCT CAC TCC GGC GGC ACT GTT GAA AGT TGT CTG GCA AAA CCT CAT A
GGA AAT CAA CAA GGT AAG ATG AGA GTG AGG TGA CAA CTT TCA ACA GAC CGT TTT GGA GTA T
SIGNAL PEPTIDE
VARIABLE REGION
SPACER
wt pIII

FIG. IA.

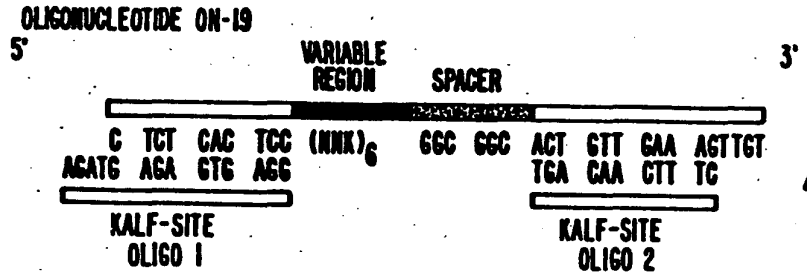
OLIGONUCLEOTIDE ON-19
5'
3'
VARIABLE REGION
SPACER
C TCT CAC TCC (NNK)6 GGC GGC ACT GTT GAA AGT TGT
AGATG AGA GTG AGG TGA CAA CTT TC
KALF-SITE OLIGO 1
KALF-SITE OLIGO 2

FIG. IB.

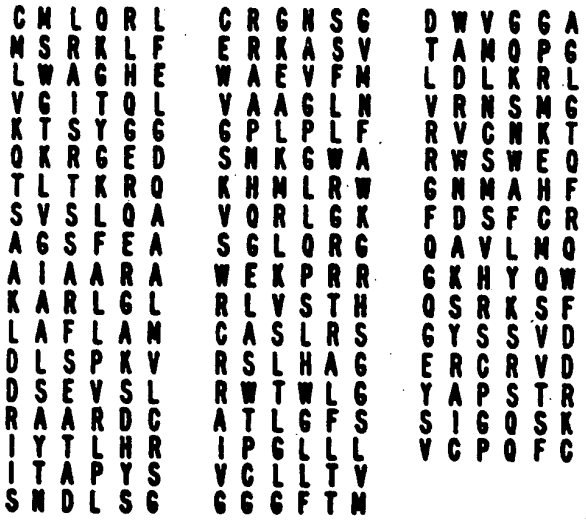

FIG. 2.

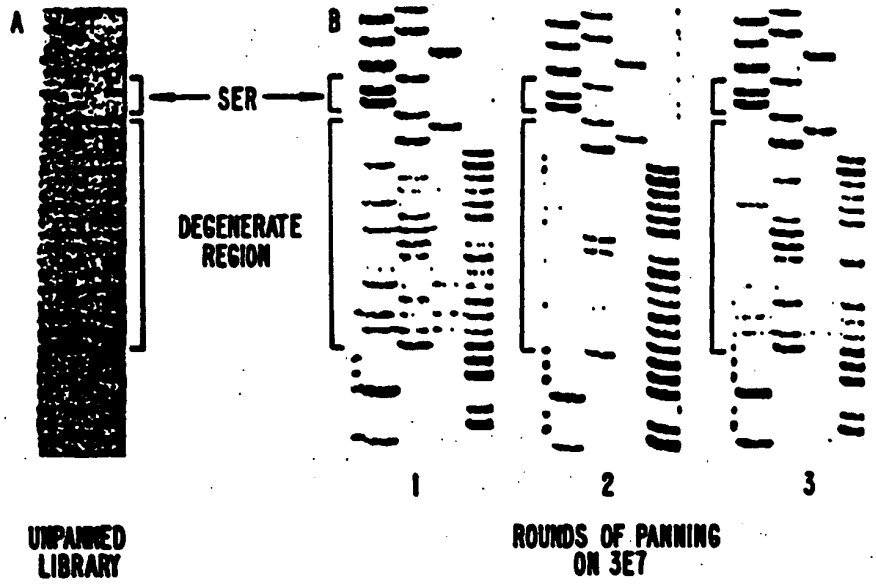
A
B
SER
DEGENERATE REGION
1
2
3
UNPANNED LIBRARY
ROUNDS OF PANNING ON 3E7

FIG. 3.

YGGLGL	YGSLVL	YGALGG	YGWWGL	YGLWQS
YGGLGI	YGSLVQ	YGALSW	YGWWLT	YGFWGM
YGGLGR	YGSLVR	YGALDT	YGWLAT	YGKWSG
YGGLMV	YGSLAD	YGALEL	YGWAMK	YGPFWS
YGGLRA	YGSLLS			YGEFVL
YGGLEM	YGSLMG	YGAIGF	YGMWTY	YGDFAF
	YGSLYE		YGMFAD	
YGGIAS		YGAWTR	YGMFPA	YAWGWG
YGGIAV	YGSWAS			YAGFAQ
YGGIRP	YGSWAS		YGTFIL	
YGGIRP	YGSWQA		YGTWST	YSMFKE
YGGWAG	YGSFLH		YGVWAS	
YGGWGP			YGVWWR	
YGGWSS				
YGGMKV				
YGGFPD				

FIG. 4.

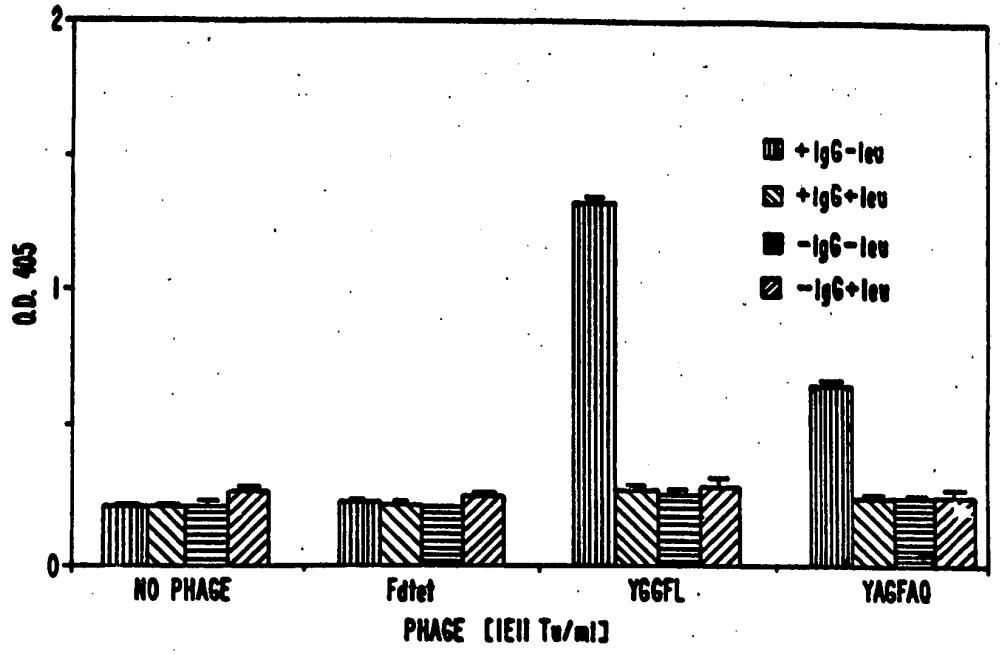
2
1
0
O.D. 405
+IgG-leu
+IgG+leu
-IgG-leu
-IgG+leu
NO PHAGE
Fdtet
YGGFL
YAGFAQ
PHAGE [IEII Tu/ml]

FIG. 5A.

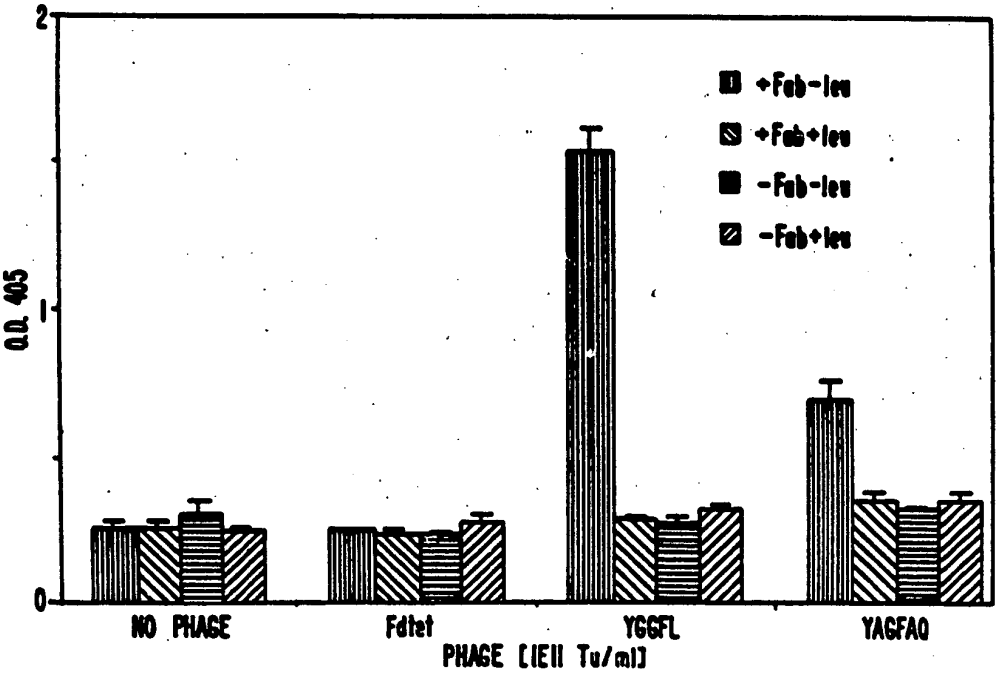
2
1
0
O.D. 405
+Fab-leu
+Fab+leu
-Fab-leu
-Fab+leu
NO PHAGE
Fdtet
YGGFL
YAGFAQ
PHAGE [IEII Tu/ml]

FIG. 5B.

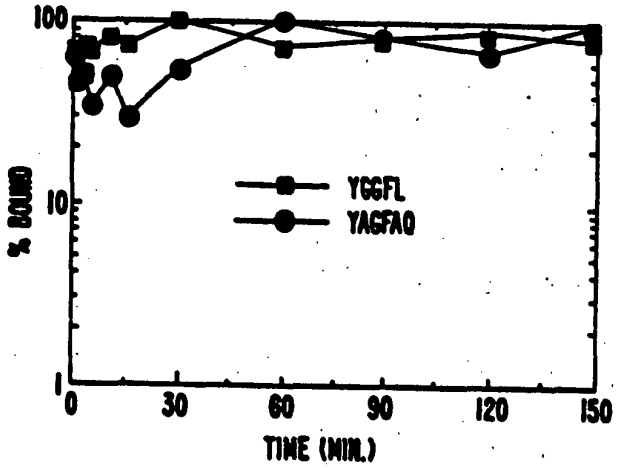
100
10
1
% BOUND
YGGFL
YAGFAQ
0
30
60
90
120
150
TIME (MIN.)

FIG. 6A.

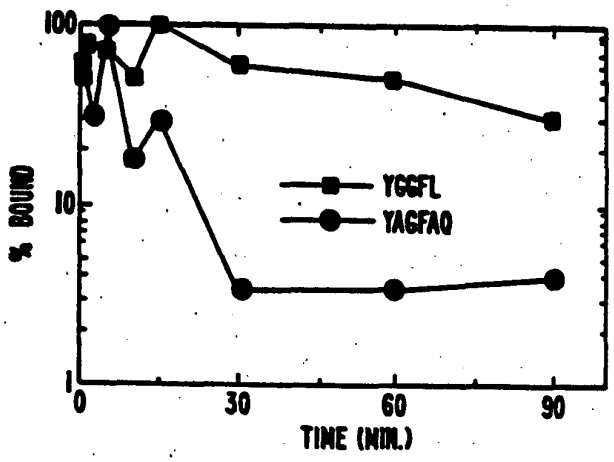
100
10
1
% BOUND
YGGFL
YAGFAQ
0
30
60
90
TIME (MIN.)

FIG. 6B.

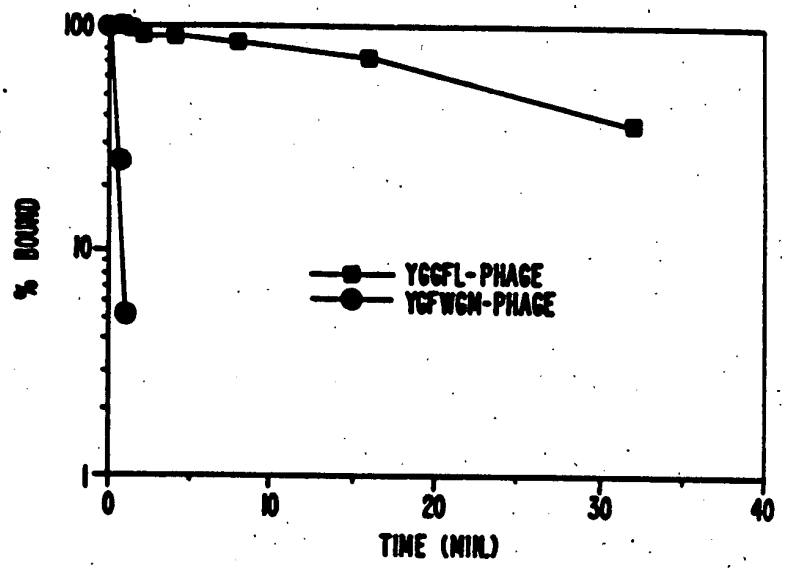

FIG. 7.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06384

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/70; A61K 37/02
U.S.CL.: 435/5; 530/300

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US.	435/5,69.1,71.1; 530/300;935/

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS Dialog

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,P	Proceedings of the National Academy of Sciences. Volume 87. issued August 1990. Cwirla et al.,"peptides on Phage: A vast Library of Peptides for Identifying Ligands," pages 6378-6382. see entire document.	1-40
A	Gene Volume 44, issued 1986. Oliphant et al..."Cloning of Random-Sequence Oligodeoxynucleotides," pages 177-183, see entire document.	1-40

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 October 1991	07 NOV 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Donna C. Wortman

Form PCT/ISA/210 (second sheet) (Rev.11-87)